

CHARLES UNIVERSITY IN PRAGUE

Faculty of Physical Education and Sport

**DAMAGE TO OLIGODENDROCYTES AND AXONS  
FOLLOWING ENDOTHELIN 1-INDUCED FOCAL  
CEREBRAL ISCHEMIA**

DIPLOMA THESIS

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UNIVERZITA KARLOVA V PRAZE

Fakulta tělesné výchovy a sportu

**POŠKOZENÍ AXONŮ A OLIGODENDROCYTŮ U  
FOKÁLNÍ MOZKOVÉ ISCHÉMIE VYVOLANÉ  
APLIKACÍ ENDOTHELINU-1.**

DIPLOMOVÁ PRÁCE

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Praha, Duben 2008

## **SUMMARY**

### **Title:**

Damage to Oligodendrocytes and Axons Following Endothelin 1-induced Focal Cerebral Ischemia

### **Aims:**

The purpose of this study was to characterize the regional patterns of neuronal and axonal injury in immature rats following FCI.

### **Methods:**

Experiments were performed in Wistar rats on postnatal day 12 and 25. FCI was induced by the intracerebral injection of Endothelin 1 (ET-1). Neuronal and axonal injury was assessed by the means of qualitative morphologic methods.

### **Results:**

Our findings demonstrate that ET-1 induced FCI in the developing rat brain generates neuronal injury in the ipsilateral cortex and a distinct pattern of subcortical neuronal injury. The spatial spread of the damage to myelinated fibers seemed to be bigger than the extent of the area, where degenerating neurons were observed.

### **Keywords:**

Oligodendrocyte, Focal cerebral ischemia, Endothelin-1, Myelin

## **SOUHRN**

### **Název:**

Poškození axonů a oligodendrocytů u fokální mozkové ischemie vyvolané aplikací Endothelinu-1.

### **Cíle práce:**

Charakterizovat poškození nervových buněk a axonů na modelu fokální mozkové ischemie v nezralé mozkové tkáni.

### **Metody:**

Experimentální studie proběhla na jedincích laboratorního potkana ve věku 12ti a 25ti dní. Fokální mozková ischemie byla vyvolána aplikací ET-1. Hodnocení poškození nervových buněk a axonů se provádělo kvalitativně popisně. Využity k tomu účelu byly morfologické metody.

### **Výsledky:**

Demonstrují, že aplikace ET-1 do nezralé mozkové tkáně potkanů, způsobuje lézi v ipsilaterálních kortikálních, ale i subkortikálních strukturách. Oblast, kde bylo nalezeno poškození myelinizovaných vláken byla většího rozsahu než oblast, kde byly lokalizovány degenerující neurony.

### **Klíčová slova:**

Oligodendrocyt, Axon, Fokální mozková ischemie, Endothelin-1, Myelin

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Prague, 3.4.2008

Carole Brožíčková

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V Praze 3.4.2006

Carole Brožíčková

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Poznámka

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## **1. INTRODUCTION**

There has been growing awareness among neurobiologists in recent years regarding the importance of understanding the molecular interactions that occur between neurons and glia in the CNS, particularly as it pertains to CNS pathological states with major impacts on society, such as trauma, stroke, and neurodegenerative diseases. It is now recognized that neurons are highly dependent on glial cells for providing structural and metabolic support constitutively, and that the extent of neurodegeneration and the degree to which neurons are protected from neurodegeneration during CNS injury and disease are influenced greatly by the activity of non-neuronal glial cells. (52)

White matter and gray matter are vulnerable to ischemic damage on human stroke, and functional deficits are generally a reflection of damage to both neuronal perikarya and their myelinated axons. (93) Oligodendrocytes, a major cellular component of white matter, are the only CNS myelin-forming cells and thus play a pivotal role in the proper execution of neural functions. (83) The myelin sheath is a multilamellar membrane structure wrapped around the axon, enabling the saltatory conduction of nerve impulses in vertebrates. (51)

Very little is known about the role of oligodendrocytes in early stage of cerebral ischemia. Interestingly, and in contrast to previous concepts (50), oligodendrocytes appear to be highly vulnerable to cerebral ischemia. (49, 88)

Axons are essential for the functioning of the nervous system, which in humans, is composed of nearly 45% white matter. (24) Axonal damage is a consequence of ischemic stroke and head injury (66,100) as well as a number of chronic neurological conditions. The mechanisms of axonal pathology are poorly understood when compared with the detailed characterization of the mechanisms that lead to neuronal perikaryal damage. (24)

In contrast to the situation in the PNS, where injured axons often regenerate successfully over long distances, axonal regeneration is minimal or absent in the adult mammalian CNS. Therefore, CNS trauma often results in severe and permanent deficits. It is now well accepted that the inability of CNS axons to regenerate is

crucially influenced by the presence of non-neuronal inhibitory factors in the axonal environment. (41)

There are several known myelin-derived proteins, which show axon growth-inhibitory properties limiting anatomical and functional recovery. Such molecules are myelin-associated glycoprotein (60, 84), oligodendrocyte-myelin glycoprotein (94), chondroitin sulfate proteoglycans (61), and the newly identified Nogo family. (60, 11, 63)

Combinations of treatments may be required to deal with the full complexity of CNS injury. Understanding the mechanisms of cell death of oligodendrocytes, and their interactions with axons, may help prevent myelin loss, and preserve neurological function. (58) In the development of neuroprotective drugs for clinical use in stroke, the efficacy of a drug, particularly with regard to functional recovery, is ultimately dependent on its ability to protect against both grey and white matter damage. (36)

## **2. THEORETICAL BACKGROUND**

### **2.1. CELLS OF THE BRAIN**

The brain, like all other organs, is made up of vast numbers of cells. Unlike some organs, however, the brain contains a wide variety of cell types. In the middle of the nineteenth century the German anatomist Rudolf Virchow recognized that cells in the brain could be divided into two distinct groups: neurons, and far more numerous group of cells that appear to surround the neurons and fill the space between them. Virchow called this second category of cell the neuroglia. (45)

Historically, glial cells were viewed as a type of central nervous system connective tissue whose main function was to provide support to the true functional cells of the brain, the neurons. This firmly entrenched concept remains virtually unquestioned for the better part of a century. But glial cells are neither connective tissue nor mere supportive cells. In contrast to early beliefs, glial cells are now recognized as intimate partners with neurons in virtually every function of the brain and as participants in the pathophysiology of the dysfunctional or diseased brain. (37)

#### **2.1.1 THE NEURON**

The neuron (nerve cell) is the fundamental unit of the nervous system. When combined into networks, neurons allow the human body memory, emotion, and abstract thought as well as basic reflexes. The human brain contains an estimated one hundred billion neurons which relay, process, and store information. (34)

Neurons have many different shapes and sizes. However, a typical neuron in a vertebrate (such as a human) consists of four major regions: a cell body, dendrites, an axon, and synaptic terminals. (34)

#### 2.1.1.1 The cell body

The cell body (soma) is the enlarged portion of a neuron that most closely resembles other cells. The entire neuron, like all other cells, is enclosed by a plasma membrane. This is a bilayer of phospholipid molecules, which acts as a barrier preventing the contents of the cell from mixing with those of the extracellular space. The plasma membrane is also an effective electrical insulator, hindering the diffusion of charged ions in and out of the cell. This is important, because signaling in nerve cells requires the controlled movement of ions across the plasma membrane, a process mediated by specialized proteins located in the membrane. (45)

The cell body contains the nucleus (which contains the genetic material, DNA) and is the site of synthesis of virtually all neuronal proteins and membranes. It also contains other organelles (for example, the mitochondria, the smooth and rough endoplasmic reticulum, the Golgiho complex, lysosomes) and it coordinates the metabolic activity of the neuron. (34, 47) In fact, because a great deal of energy is required to maintain the transmembrane ionic gradients that are essential for neuronal signaling, neurons tend to be particularly rich in mitochondria. There are also ribosomes, which are responsible for the synthesis of proteins destined for insertion into membranes, or for secretion. They are located on the membranous sacs of the rough endoplasmic reticulum. (45)

#### 2.1.1.2 The dendrites

The dendrites, which branch out in treelike fashion from the cell body, are specialized to receive signals and transmit them toward the cell body. The single long axon carries signals away from the cell body. (34)

#### 2.1.1.3 The axons

##### *Structure of axons*

Axons are essential for the functioning of the nervous system, which in humans, is composed of nearly 45% white matter. (24)

Almost every neuron has a single axon, whose diameter varies from a micrometer in certain nerves of the human brain to a millimeter in the giant fiber of the squid. (47)

The axon originates at a cone shaped thickening on the cell body called the axon hillock. It is often (but not always) unbranched until just before it terminates, but it may branch many times in its terminal region. The diameter of the axon remains more or less unchanged throughout its length. Its structure, like that of the dendrite, is formed and maintained by the cytoskeleton, cellular scaffolding that is present in all cells. (45)

### **Action potentials**

Axons are specialized for the conduction of a particular type of electric impulse, called action potentials. These are propagated from the cell body down the axon to the synaptic terminals. (34)

An action potential is a series of sudden changes in the voltage, or equivalently the electric potential, across the plasma membrane. When a neuron is in the resting (nonstimulated) state, the electric potential across the axonal membrane is approximately  $-60$  mV (the inside negative relative to the outside); the magnitude of this resting potential is similar to that of the membrane potential in most non-neuronal cells. (47)

When the membrane potential is less negative than the resting potential, the cell is said to be depolarized. As the depolarizing stimulus gets larger, a critical stimulus strength or threshold is reached. The level of the threshold varies from neuron to neuron, but it tends to be in the range 10-20 mV depolarized from the resting potential. Beyond the threshold, one observes a large change in membrane potential several milliseconds in duration. The membrane potential depolarizes very rapidly. (45)

The depolarization of the membrane is followed by a rapid repolarisation, returning the membrane potential to the resting value. These characteristics distinguish an action

potential from other types of changes in electric potential across the plasma membrane and allow an action potential to move along an axon without diminution. (47)

Only stimuli of sufficient importance result in information transfer via action potentials in the axon. Another important property of action potentials is that they are all-or-none events. The all-or-none law means that any stimulus large enough to produce an action potential produces the same size action potential, regardless of the stimulus strength. In other words, once the stimulus is above threshold, the amplitude of the response no longer reflects the amplitude of the stimulus. (45)

The phenomenon of passive spread (as will be discussed in more details later), allows the propagation of action potential along the axon and the transfer of information over long distances within the neuron.

Action potentials move rapidly, at speeds up to 100 meters per second. In humans, axons may be more than a meter long, yet it takes only a few milliseconds for an action potential to move along their length. A single axon in the central nervous system can synapse with many neurons and induce responses in all of them simultaneously. (47)

### **Axonal transport**

The axon utilizes a process known as axonal transport for moving vesicles and other organelles to regions remote from the neuronal cell body. Proteins called molecular motors make use of energy released by hydrolysis of ATP to drive axonal transport (45)

By a process called anterograde transport, vesicles or multiprotein particles are transported along microtubules down the length of the axon to the terminals, where they are inserted into the plasma membrane or other organelles. Axonal microtubules also are the tracks along which damaged membranes and organelles move up the axon toward the cell body; this process is called retrograde transport. (47)

We have seen that cutting an axon, or interrupting transport in axon, produces a restructuring of the synaptic contacts made by that axon and others that innervate the same target. These same procedures can also produce changes in the presynaptic contact onto the cell whose axon has been cut. The loss of presynaptic terminals following

interruption of an axon has been observed in many different pathways (56). It appears, therefore, that the normal maintenance of synaptic endings may depend on factors available only from an intact postsynaptic cell. (45)

### **Axonal regeneration in the CNS of mammals**

The capacity for injured neurons to regenerate axons and remake functional connections with target organs distinguishes the peripheral from the central nervous system. This unique characteristic of the peripheral nervous system stems from the ability of the Schwann cells as opposed to the inability of the oligodendrocytes in the central nervous system to support axonal regeneration. In consequence, return of function after peripheral nerve injuries contrasts with permanent deficits associated with central nerve injuries. (65)

After a lesion develops, the CNS environment contains different cell types that could be involved in the expression of such inhibitory molecules: oligodendrocytes, astrocytes, activated microglia and fibroblasts. All of these cell types have now been shown to express or produce inhibitory factors that can influence axonal outgrowth. (41)

There are several known myelin-derived proteins, which show axon growth-inhibitory properties limiting anatomical and functional recovery. Such molecules are myelin-associated glycoprotein (MAG) (60, 84), oligodendrocyte-myelin glycoprotein (OMgp) (94), chondroitin sulphate proteoglycans (61), and the newly identified Nogo family (16i, 11). Current approaches strive to neutralize the inhibitory action of Nogo-A by administration of purified anti-Nogo-A-antibodies via miniosmotic, subcutaneously implanted pumps that deliver the antibody directly into the cerebrospinal fluid. An important future approach to neutralize the action of growth inhibitory molecules including the Nogo family, MAG, and OMgp is the administration of receptor antagonists and antibodies. (23, 46)



#### 2.1.1.4 The synapses

Synapses generally transmit signals in only one direction: an axon terminal from the presynaptic cell sends signals that are picked up by the postsynaptic cell. The postsynaptic neuron at certain synapses also sends signals to the presynaptic one. Such retrograde signals can be gases, such as nitric oxide and carbon monoxide, or peptide hormones. This type of signaling, which modifies the ability of the presynaptic cell to signal the postsynaptic one, is thought to be important in many types of learning. (47)

There are two general types of synapse: the relatively rare electrical synapse, and the chemical synapse. Chemical and electrical synaptic transmission exists side by side in most nervous systems.

##### *The electrical synapses*

There are no morphological specializations that allow pre- and postsynaptic elements to be distinguished in this type of synapses. Indeed since signals can move in both directions through electrical synapses, each cell may be pre- or postsynaptic at different times. Electrical synapses are characterized by an area of very close apposition between the membranes of the pre- and postsynaptic cells. Within these membranes are found gap junctions. (45)

The channels comprising a gap junction are large-diameter aqueous pores that extend from one cell, across the extracellular space, into an adjacent cell. These channels allow all inorganic ions and small organic molecules to diffuse freely from the interior of one cell into the interior of another cell. (74)

Two important functional properties follow immediately from this mode of transmission. First, as referred to above, information transfer can be bidirectional. The second important functional consequence of this mechanism is that electrical synapses are fast. There is no delay analogous to that seen with chemical synaptic transmission. (45)

The discovery of gap junctional communication challenged the fundamental concept of classical cell theory, which states that individual cells are autonomous functional units. When cells are connected with their neighboring cells by gap junctions, the functional unit is no longer the individual cell of classical cell theory but rather the large aggregate of cells that are so joined, because they share so much of their cytoplasmic contents. (74)

### **Chemical synapses**

In this type of synapse, the axon terminal of the presynaptic cell contains vesicles filled with a particular neurotransmitter. The postsynaptic cell can be a dendrite or cell body of another neuron, a muscle or gland cell, or, rarely, even another axon. When an action potential in the presynaptic cell reaches an axon terminal, it induces a localized rise in the level of  $\text{Ca}^{2+}$  in the cytosol. This, in turn, causes some of the vesicles to fuse with the plasma membrane, releasing their contents into the synaptic cleft, the narrow space between the cells. The neurotransmitters diffuse across the synaptic cleft; it takes about 0.5 milliseconds (ms) for them to bind to receptors on postsynaptic cells.

Binding of the neurotransmitter triggers changes in the ion permeability of the postsynaptic plasma membrane, which, in turn, changes the membrane's electric potential at this point. If the postsynaptic cell is a neuron, this electric disturbance may be sufficient to induce an action potential. If the postsynaptic cell is a muscle, the change in membrane potential following binding of the neurotransmitter may induce contraction; if a gland cell, the neurotransmitter may induce hormone secretion. (47)

### **Glia and synaptic transmission**

Glial cell processes envelop many synapses in the CNS and the PNS, often abutting the synaptic cleft. This close structural association suggests a physiological interaction between glial cells and neurons. Indeed, glial cells possess many neurotransmitter

receptors and respond actively to a variety of neurotransmitters and modulators. Activated glial cells, in turn, release chemical transmitters that stimulate postsynaptic neurons and modulate transmitter release from presynaptic terminals. (59)

These close structural and physiological interactions have led to the suggestion that glial cells function as an active partner in synaptic transmission. The importance of glial cells in synaptic function has been emphasized by the coining of the term tripartite synapse to stress that the third element of the synapse, the glial cell, is an essential constituent in determining synaptic function, along with the pre- and postsynaptic neurons. (59)

Few, if any, gap junctions are found between oligodendrocytes in white matter of the cat, although gap junctions are abundant between oligodendrocytes and astrocytes. Based on ultrastructural data, it is proposed that communication between oligodendrocytes is mediated by connections through the astrocyte syncytium. (75) However, gap junctions have been seen between purified rat oligodendrocytes, and physiological coupling can be demonstrated directly between oligodendrocytes in vitro and in situ. (74) Oligodendrocytes appear to be widely coupled by junctions that allow only weak electrical interaction, in accordance with the ultrastructural observation that interoligodendrocytic gap junctions are much less frequent than gap junctions between astrocytes. (75)

## 2.1.2 OLIGODENDROCYTES

### 2.1.2.1 Morphology and function of oligodendrocytes

RioHortega (1928) is credited with first identifying oligodendrocytes as process-bearing cells by metal impregnation techniques. The oligodendrocyte is mainly a myelin forming cell, but there are also satellite oligodendrocytes that may not be directly connected to the myelin sheath. Satellite oligodendrocytes are perineuronal and may serve to regulate the microenvironment around neurons. (48)

An oligodendrocyte extends many processes, each of which contacts and repeatedly envelopes a stretch of axon with subsequent condensation of this multispiral membrane-forming myelin. On the same axon, adjacent myelin segments belong to different

oligodendrocytes. The number of processes that form myelin sheaths from a single oligodendrocyte varies according to the area of the CNS and possibly the species. (5)

Rio Hortega classified oligodendrocytes in four categories, in relation to the number of their processes. According to their morphology and the size or thickness of the myelin sheath they form, Butt et al. also distinguish four types of myelinating oligodendrocytes, from small cells supporting the short, thin myelin sheaths of 15–30 small diameter axons (type I), through intermediate types (II and III), to the largest cells forming the long, thick myelin sheaths of one 1–3 large diameter axons. (5)

These phenotypic differences determine the conduction properties of axons within oligodendrocyte units. The numbers and distributions of oligodendrocyte phenotypes are related to the age of myelination, and are likely to be determined by the competition for axon-derived and environmental factors. Key issues are how environmental, axonal, and genetic factors control the differentiation of oligodendrocyte phenotypes and how oligodendrocytes and myelin, in turn, determine axonal growth and integrity, as well as the periodicity of nodes of Ranvier (will be discussed later). (10)

#### 2.1.2.2 Types of oligodendrocyte cell death

Oligodendrocyte cell death can follow necrotic, apoptotic, or hybrid pathways. In general, feature of necrotic cell death include loss of cell membrane integrity, rapid organelle swelling, mitochondrial failure, and energy depletion. Apoptotic features include nuclear condensation, intranucleosomal DNA cleavage, and membrane blebbing. In hybrid cell death, cells present features of both apoptosis and necrosis, which is hypothesized to result from the initiation of apoptosis followed by energy failure and necrotic mitochondrial and plasma membrane rupture. (53)

In vitro and in vivo experiments have shown that oligodendrocytes are very vulnerable to hypoxic and ischemic insults. What triggers the death of oligodendrocytes in cerebral ischemia remains to be elucidated. (83)

Recent evidence indicates that excitotoxic mechanisms can also contribute to oligodendrocyte injury. Initial cell culture studies defining oligodendrocyte sensitivity

to glutamate demonstrated that glutamate-mediated cell death of oligodendrocyte lineage cells is elicited by activation of either AMPA or kainate receptors, but not by NMDA or metabotropic glutamate receptors, in a dose-dependent manner. (58)

Myelin damage in the CNS and PNS is frequently associated with immune and inflammatory disorders. Oligodendrocyte and Schwann cell injury may be mediated by cytokines, antibody, complement, or specific activation of death receptors. Immune cells, microglia, and reactive astrocytes are all sources of cytokines in the damaged CNS. (58)

### 2.1.2.3 Oligodendrocytes and myelin

The myelin sheath around most axons constitutes the most abundant membrane structure in the vertebrate nervous system. Its unique composition (richness in lipids and low water content allowing the electrical insulation of axons) and its unique segmental structure responsible for the salutatory conduction of nerve impulses allow the myelin sheath to support the fast nerve conduction in many fibers in the vertebrate system. (1M) The sheath is formed by OCs in CNS axons and by Schwann cells in the PNS. A single Schwann cell myelinates only one axon, in contrast to oligodendrocytes, which myelinate a family of axons, with estimates ranging from 1-2 to nearly 100 axons per family (45)

The importance of this myelin covering to normal nervous system function is made painfully obvious in individuals with demyelinating diseases in which the myelin covering of the axons is destroyed. Among these diseases is multiple sclerosis, a demyelinating disease of the central nervous system that can have devastating consequences, including visual, sensory, and motor disturbances. (34).

#### **Structure and composition of myelin**

When a myelinated axon is examined in cross section with an electron microscope, the axon is found to be surrounded by concentric circles of alternating dark and light bands. This structure arises by the tight wrapping of the membrane of the oligodendrocyte or

Schwann cell around the axon during development. The cytoplasm of the glial cell is gradually squeezed out of this region as the cell wraps at around the axon, so that the concentric circles represent layers of closely apposed glial plasma membrane. (45)

In contrast to most plasma membranes, myelin is a lipid-rich membrane (lipids constitute 70% of dry myelin weight) that is highly enriched in glycosphingolipids and cholesterol. There is also an unusually high proportion of ethanolamine phosphoglycerides in the plasmalogen form, which accounts for one-third of the phospholipids. Myelin contains a relatively simple array of proteins, myelin basic protein (MBP) and the proteolipid proteins (PLP/DM20) being the two major CNS myelin proteins. (57).

During the active phase of myelination, each oligodendrocyte must produce as much as  $\approx 5\text{-}50 \times 10^3 \mu\text{m}^2$  of myelin membrane surface area per day. (72)

### **Saltatory conduction**

The speed of action potential propagation along the axon is determined in part by myelination. This comes about, because the myelin sheath, which consists of a large number of layers of glial plasma membrane wrapped about the axon, acts as an excellent electrical insulator. The high electrical resistance and low capacitance of myelin prevents current loss during action potential conduction. (35, 45)

The myelin is punctuated by nodes of Ranvier. The internode distances ranging from less than 100 $\mu$  (small-diameter fibers) to slightly over 1mm (larger-diameter fibers) optimize conduction velocity. Because of their specialization, both structural and molecular, myelinated fibers can conduct action potentials rapidly in a discontinuous or saltatory manner, unlike nonmyelinated fibers, which usually conduct impulses in a slower, continuous manner. (35)

As already mentioned, the saltatory conduction (from the Latin *saltare*, meaning to leap) permits conduction at speeds many time faster than in nonmyelinated axons of the same diameter. The passive spread of the action potential depolarization along the myelinated portion of the axon can bring the adjacent node above threshold, allowing the action potential to „jump“ along the axon from node to node.

Myelinated axon membrane is highly specialized, with several types of voltage-sensitive ion channels and other proteins distributed in a spatially heterogeneous manner. The sodium channels are not spread evenly throughout the axonal plasma membrane, but are sparse in the intervening membrane under myelin. (45) At the nodes of Ranvier,  $\text{Na}^+$  channels cluster in high density in the axon membrane. Together these factors allow the action potential to jump from node to node, achieving conduction with the minimum use of ion channels or energy-consuming pumps.

The high density of nodal  $\text{Na}^+$  channels and the paucity of these channels in the axon membrane under the myelin, combined with the presence of fast  $\text{K}^+$  channels in the axon membrane under the paranodal myelin, have important implications for axonal pathophysiology. (35)

The need for rapid conduction of the nerve impulse serves as a driving force that can determine and increase animal size. For an axon without myelin, the speed of impulse conduction is proportional to the diameter<sup>1/2</sup>. Therefore, in order to achieve a faster rate of conduction, species that lack myelin have to substantially enlarge their axons. (35)

### **Myelin biogenesis**

Myelination is a developmentally regulated event that begins postnatally in the rat and mouse brain and within the third fetal trimester in the human spinal cord. (12) In those species in which birth occurs before myelination, the newborns are extremely limited in motor performance, and in fact are quite helpless until myelination is complete. (45)

Since the ensheathment of the axons must occur at the appropriate time of neuronal development, reciprocal communication between neurons and oligodendrocytes is essential to coordinate myelin biogenesis. (7) Neurons control the development of oligodendrocytes by regulating the proliferation, differentiation and survival of oligodendrocytes. (4) The signals are important to match the number of oligodendrocytes to the axonal surface requiring myelination. Furthermore, the timing of myelination is crucial because the ensheathment of axons must not occur before neurons signal to oligodendrocytes. In turn, signals from oligodendrocytes to neurons are necessary to cluster multiprotein complexes in the axonal membrane into distinct

subdomains at the nodes of Ranvier. (57) Moreover, the axonal cytoskeleton and the rate of vesicular transport along the axons are modified by oligodendrocytes. (20)

Myelination of the axonal tracts of the CNS by oligodendrocytes takes place when oligodendrocytes have differentiated from oligodendrocyte precursor cells (OPCs). OPCs arise from the neuroepithelium of ventricular/subventricular zone of the brain and migrate from this region into the developing white matter until they reach the appropriate axons. After OPCs have arrived at their final target, they exit the cell cycle, become non-migratory and differentiate into myelin forming oligodendrocytes. To ensure full and timely myelination of all axonal tracts, the timing of OPC differentiation must be tightly controlled by their target cells. (57)

Many studies now point to the importance of extrinsic neuron-derived signaling molecules at multiple stages of oligodendrocyte development. (13M) these extrinsic signals serve two major purposes. They help to control the proper timing of OPC differentiation to ensure myelination at the appropriate time and place, and they control and match the number of oligodendrocytes to the axonal surface area requiring myelination. Several growth factors and trophic factors, such as PDGF-A, FGF-2, IGF-1, NT-3 and CNTF, have been shown to regulate oligodendrocyte development. (57)

The next event after differentiation of OPCs is the formation of myelin. Myelination is a multi-step process requiring precise coordination of several different signals. The following steps can be distinguished:

- Recognition of and adhesion of the oligodendrocytes to the appropriate axon
- Synthesis and transport of myelin components to the axon
- The wrapping of the myelin membrane around the axons
- The compaction of the myelin sheath. (57)

The crucial mechanism by which an oligodendrocyte ensheaths axons with a spiral wrap that finally compacts to form the myelin sheath is a fundamental aspect of both myelination and remyelination about which little is known. (25)

### **Demyelination**



When the myelin is damaged, the density of action current falls due to capacitative and resistive shunting. If the safety factor (the ratio between current available to stimulate a node of Ranvier and current required to stimulate the node) is reduced, but is still greater than 1, the charging time for the nodal membrane will be increased so that it will take longer than normal for the axon to reach threshold, and conduction will continue but conduction velocity will be reduced. In more severely demyelinated axons, the safety factor can fall under 1, so that threshold will not be reached, hence conduction will fail.

In addition to capacitative current loss through injured myelin sheaths, conduction in focally demyelinated axons can fail due to mismatch. The low density of Na<sup>+</sup> channels in the demyelinated axon membrane also contributes to conduction block. Moreover, fast K<sup>+</sup> channels are unmasked after demyelination, and due to that they impede action potential electrogenesis. (57)

The pathogenesis of demyelination after injury may be due to loss of mature oligodendrocytes or may be secondary to other processes such as microglial activation or loss of trophic support after axonal degeneration. Some evidence suggests that insulin-like growth factor-1 (IGF-1) may reduce primary and secondary postischemic white matter injury. IGF-1 promotes the proliferation and differentiation of oligodendroglia and up-regulates myelin production *in vitro*. IGF-1 has broad, receptor-mediated antiapoptotic effects *in vitro* and *in vivo* and specifically inhibits the apoptotic loss of oligodendrocytes associated with cytokine toxicity and metabolic insults. Experimental demyelination is associated with distinctive patterns of induction of IGF-1 in astrocytes and the IGF-1 receptor in oligodendrocytes during subsequent regeneration (33), suggesting that endogenous IGF-1 may play a key role in remyelination.

Currently, there is little information on the role of IGF-1 in oligodendrocyte survival or cerebral demyelination after hypoxic–ischemic injury in the developing brain. It has been shown that postischemic administration of IGF-1 can protect cortical and striatal neurons from ischemic injury in the near-term fetal sheep. (32)

## 2.2 THE CEREBRAL CORTEX

The cerebral cortex is well endowed with neurons, neuroglia, nerve fibers, and a rich vascular supply. The arrangement of the three types of neurons that populate the cortex - pyramidal cells, stellate neurons, and fusiform neurons - permit the classification of the cortex into three types: the archicortex (allocortex), mesocortex (juxtallocortex), and neocortex (isocortex).

The archicortex, phylogenetically the oldest region, is composed of only three layers and is located in the limbic system. The mesocortex, phylogenetically younger, is composed of three to six layers, and is located predominantly in the insula and cingulate gyrus. The neocortex, phylogenetically the youngest region of the cerebral cortex, is composed of six layers and comprises the bulk of the cerebral cortex. (31)

### 2.2.1 THE NEOCORTEX

The neocortex is that part of the brain which makes up the outer 2 to 4 mm of the cerebral hemispheres. It is the 'gray matter' of the brain lying atop the cerebral 'white matter' composed of myelinated axons that interconnect different regions of the brain. (96) The corpus callosum connects the neocortex of the right hemisphere with that of the left. (31)

All the higher-level psycho-physical functions sensory perception, object- and event-representation, planning, and decision making are believed to take as their biological substrate the activities of interconnected and distributed networks of neurons in the neocortex. (96)

Glia and non-neuroglia elements make up almost 70% of the volume of the neocortex. Of the remainder, 22% consists of axons and dendrites, with the body (soma) of the neuron comprising only 8%. (76)

The neocortex can vary in thickness and topology. In comparison of the human and rat cortices we can see a definite difference in shape. The wave like nature of the cortex we see in the human brain is not as apparent in the brain of the rat. (9)

The cortex structure is highly folded with many grooves (called 'sulci'). This folded arrangement allows for a far greater volume of cortical matter to be contained within a given-sized brain cavity than would be possible if the cortex were laid out in a 'sheet' directly beneath the skull. The sulci provide convenient 'landmarks' for helping anatomists to classify different regions of the cerebral cortex. (96) The defined areas of the cortex include the motor cortex, the sensory cortex and the associative cortex. All three of these areas are responsible for functions of the organisms ranging from control of body movement to sound recognition to mental processing. (9)

#### 2.2.1.1 Cell types and layers of the neocortex

As was said, the cortex depth can be various depending on the animal and consists of approximately 6 layers. The layers can be distinguished by the horizontal nature of the cell geometries and/or densities that are particular to each layer. (9)

Layer I, the outermost layer, is often called the molecular layer or, somewhat misleadingly, the 'acellular' layer. (96) Layer I, however, actually contains few neurons and is mostly made up of tangentially running axons and horizontally running bifurcating apical dendrites received from the pyramidal cells of the lower layers. (76)

Layer II, the external granular cell layer, contains a mix of small pyramidal cells and some inhibitory neurons, mainly bipolar cells and double bouquet cells. It also contains apical dendrites from pyramidal cells whose cell bodies are found in layers V and VI.

Layer III contains a variety of cells essentially consisting of almost all cell types found in the neocortex except the excitatory spiny stellate cell and cells found exclusively in layer I (the Cajal-Retzius cell and small unclassified inhibitory cells). The majority of cells in layer III are small pyramidal cells. (96)

Layer IV, the Internal Granular Layer, has a granular appearance and consists of small pyramidal, granule, and stellate (star shaped) cells and receives massive axonal projections from the thalamus. These neurons are predominantly local circuit, and project to adjacent columns and layers. That is, upon receiving and analyzing thalamic input, the neurons of layer IV transfer this data to adjacent neurons for additional

analysis. Because the primary, secondary and association sensory areas receive considerable thalamic input, layer IV is relatively thick- except in the motor cortex. (76)

Layer V is composed mainly of large pyramidal cells with a smaller population of inhibitory cells. Axons and possibly basal dendrites of non-spiny bipolar cells (which are inhibitory) are also found in layer V. Chandelier cells, which are inhibitory cells that make synaptic connections only to the axons protruding from other neurons, are often found in layer V. (96) The pyramidal neurons of layer V are long distance neurons, and give rise to descending axons which form the corticospinal, pyramidal, corticobulbar, corticopontine, and corticorubral brainstem pathways which establish contact with cranial nerve and sensory and spinal motor neurons. (76)

Layer VI is a heterogeneous layer of various neurons that blends gradually into the white matter. Most of the cells in layer VI are large pyramidal cells that project their axons back to the thalamus. Layer VI also contains a class of inhibitory neurons called Martinotti cells whose axonal outputs make long projections across all layers of the neocortex. After layer IV, layer VI is the next principal target of thalamic inputs to the neocortex. (96)

## **2.3 THE HIPPOCAMPAL FORMATION**

The hippocampal formation is a prominent component of the rat nervous system that has attracted the attention of neuroanatomists since the beginning of formal study of the nervous system. (1) The hippocampus is perhaps the most studied structure in the brain. Together with the adjacent amygdala, it forms the central axis of the Limbic System. (See att. 1)

### **2.3.1 ANATOMY AND CONNECTIONS OF THE HIPPOCAMPAL FORMATION**

The term hippocampal formation is commonly used to refer to the hippocampus proper and dentate gyrus, although it has to be acknowledged that the term is sometimes also used to include the subicular complex and sometimes even the entorhinal cortex. (1)

Ramón y Cajal (1893) divided the hippocampus into two components: dentate gyrus and Ammons horn, and Ammons horn was subdivided in an “upper” (also labeled regio superior) and a “lower” region (regio inferior). This terminology has largely been displaced by the terminology that was introduced by Lorente de Nó, i.e., dentate gyrus and three subfields (CA1 [regio superior], and CA2 and CA3 [regio inferior]). But, especially in human and monkey literature the terms regio superior and regio inferior, and CA4 for hilus, are still used. (39)

#### 2.3.1.1 Dentate gyrus

The major input is from enthorhinal cortex via the so-called perforant path. The entorhinal to dentate gyrus projection is not reciprocated. (1)

The dentate gyrus consists of three layers: the molecular layer, the granule cell layer, and the polymorph zone. The principal cells of the dentate gyrus are the granule cells, whose dendritic tree is confined to stratum moleculare. The hilus, or polymorph zone of the dentate gyrus, is located below the granule cell layer and is bordered by CA3. The principal, and most abundant, cell types of the hilus are the mossy and basket cells. But many other interneurons are present in the dentate gyrus and hilus and these have projections that, in general, are related to the type of interneuron, e.g., basket cells primarily innervate the cell body layer. The granule cells of the dentate gyrus are considered to be the first stage of the trisynaptic loop. (1, 39)

#### 2.3.1.2. Hippocampus proper

The axons from the granule cells (the mossy fibers) leave the hilar region and synapse on the proximal dendrites of the CA3 pyramidal cells, and this represents the second stage of the trisynaptic loop. It should be noted that the mossy fibers also terminate densely on the mossy and basket cells of the hilus. The basal dendrites of the CA3, CA2, and CA1 pyramidal cells ramify in stratum oriens above the pyramidal cell layer, whereas the apical dendrites are present below the pyramidal cell layer. The area where the shafts of the apical dendrites are located (with little branching) is called stratum

radiatum, and the apical dendrites branch extensively in stratum lacunosum-moleculare. (39)

In CA3 there is an additional layer called stratum lucidum located between the pyramidal cell layer and the stratum radiatum, within which the mossy fibers course and terminate. The axons of CA3 pyramidal cells project densely to CA1, as originally described by Schaffer (1892), but they also give rise to relatively dense axonal arborisations within CA3. The projection to CA1 represents the third stage of the trisynaptic loop. (39)

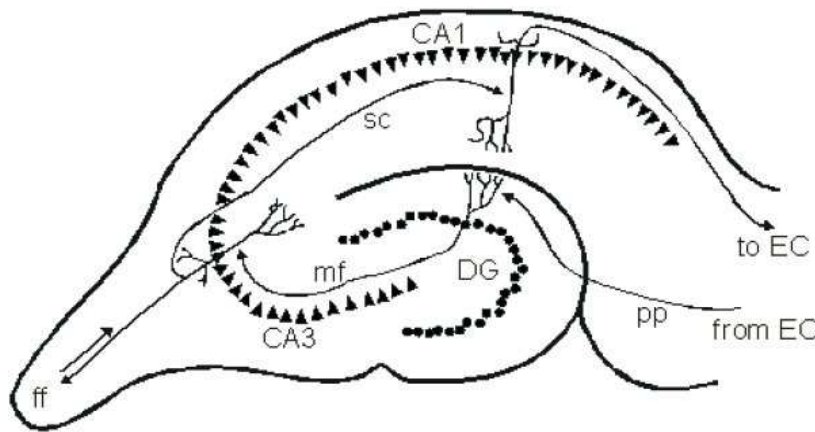
#### 2.3.1.3. Subiculum

The axons from the CA1 pyramidal cells synapse on the dendrites of the subicular pyramidal cells and this can be seen to represent the “fourth” stage of the trisynaptic loop. Unlike the pyramidal cells of CA3 and CA1, the pyramidal cells of the subiculum are not arranged in one layer, they are more dispersed and show differences between the deeper and more superficial neurons. (39)

The subiculum has a range of electrophysiological and functional properties which are quite distinct from its input areas; given the widespread set of cortical and subcortical areas with which it interacts, it is able to influence activity in quite disparate brain regions. The rules governing plasticity of synaptic transmission in the hippocampal-subicular axis are poorly understood; this axis appears to share some properties in common with the hippocampus proper, but behaves quite differently in other respects. Equally, its functional properties are not well understood; it plays an important but ill-defined role in spatial navigation, mnemonic processing and control of the response to stress. (64)

#### 2.3.1.4 The trisynaptic loop

The hippocampus, when cut transverse to its longitudinal (septal-temporal) axis, exhibits a strong afferent set of three connected pathways known as the "trisynaptic" circuit or loop. (97)



**Figure 1.** The trisynaptic loop of the hippocampus. The filled triangles represent the pyramidal cell layer (CA1 and CA3) and the filled circles represent the granular cell layer of the dentate gyrus. Abbreviations: EC = entorhinal cortex; DG = dentate gyrus; pp = perforant pathway; mf = mossy fibers; sc = Schaffer collaterals; ff = fimbria fornix. (1)

Afferents from the entorhinal cortex synapse onto granule cells, the principal cells of the dentate gyrus, via the perforant-path. The granule cells send their axons (mossy fibers) to the proximal dendrites of CA3 pyramidal cells. In turn, these cells project to the dendritic regions of CA1 pyramidal cells via Schaffer collaterals. The principle extrinsic projections arise from the CA1 pyramidal cells and terminate in the entorhinal cortex and subiculum, completing the cortico-hippocampo-cortical loop. (95)

## **2.4. STROKE**

### **2.4.1 INTRODUCTION TO STROKE**

A stroke occurs when the blood supply to part of the brain is suddenly interrupted or when a blood vessel in the brain bursts, spilling blood into the spaces surrounding brain cells. Brain cells die when they no longer receive oxygen and nutrients from the blood or there is sudden bleeding into or around the brain. The symptoms of a stroke include sudden numbness or weakness, especially on one side of the body; sudden confusion or trouble speaking or understanding speech; sudden trouble seeing in one or both eyes; sudden trouble with walking, dizziness, or loss of balance or coordination; or sudden severe headache with no known cause. There are two forms of stroke: ischemic - blockage of a blood vessel supplying the brain, and hemorrhagic - bleeding into or around the brain. (62)

### **2.4.2 STROKE STATISTICS**

Stroke is the leading cause of disability in the United States. Approximately 700,000 people experience a stroke each year. There are an estimated 5 million stroke survivors living in the

United States. Stroke places a financial burden on our society with the projected cost of stroke care in 2007 approaching 62 billion dollars. (77)

The most significant physical impact on stroke survivors is long term disability. In the stroke survivor population, 50% have some level of hemiparesis, 30% are unable to walk without some assistance and 20% are dependent in activities of daily living. (77)

Men have strokes at a younger age than women; therefore the age adjusted incidence of stroke is 1.25 times greater for men than for women. (2) The risk of stroke for blacks is almost double that of Whites, and Hispanics have a greater incidence of hemorrhagic stroke at a younger age. Most strokes (88%) are ischemic events, while other etiologies include intracerebral haemorrhage (9%) and subarachnoid haemorrhage (3%).



Stroke is a life changing event that affects not only the person who may be disabled, but the entire family and other caregivers as well. Utility analyses show that a major stroke is viewed by more than half of those at risk as being worse than death. (80)

### 2.4.3 RISK FACTORS OF STROKE

The major or more common risk factors for cerebral ischemia and infarction include family history, hypertension, smoking tobacco, diabetes mellitus, higher body mass index, and other risk factors for the development of atherosclerosis such as hypercholesterolemia.

Smoking increases the risk of a stroke due to either arterial wall damage and atherosclerosis or formation and rupture of aneurysms. (69) Virtually every multivariable assessment of stroke risk factors (eg., Framingham, Cardiovascular Health Study, and the Honolulu Heart Study) has identified cigarette smoking as a potent risk factor for ischemic stroke associated with an approximate doubling of ischemic stroke risk (after adjustment for other risk factors). There is growing acceptance that exposure to environmental tobacco smoke (passive cigarette smoke) is a risk factor for heart disease. Several studies suggest that environmental tobacco smoke is also a substantial risk factor for stroke, with a risk approaching the doubling found for active smoking. (30, 101)

Cardiac risk factors of stroke include atrial fibrillation and a recent myocardial infarction. (69) Other types of cardiac disease that can contribute to the risk of thromboembolic stroke include dilated cardiomyopathy, valvular heart disease (eg. mitral valve prolapse, endocarditis, prosthetic cardiac valves), and intracardiac congenital defects (eg. patent foramen ovale, atrial septal defect, atrial septal aneurysm). (30)

The risk of hemorrhagic stroke is increased in persons of Asiatic or African descent. (69) Racial and ethnic effects on disease risk can be difficult to consider separately. African Americans (79, 99, 8) have higher stroke incidence and mortality rates as compared with European Americans. In the Atherosclerosis Risk in Communities Study, blacks had an incidence of stroke 38% higher than that of whites. (78) Possible

reasons for the higher incidence and mortality rate of strokes in blacks include a higher prevalence of hypertension, obesity, and diabetes within the black population. (27) Epidemiological studies have shown an increase in stroke incidence among self-identified Hispanic racial-ethnic populations. (79, 26) Incidence rates are also relatively higher among some Asian groups. (86)

Evidence of a prior TIA also increases the risk of a stroke. Additionally, the risk of having a stroke increases with age. (43) The use of hormonal contraceptives can also increase the risk of cerebral ischemia or infarction.

Other risk factors include hypertension, trauma, advanced age, heavy alcohol consumption, and cocaine and amphetamine abuse. (2)

A sedentary lifestyle is associated with an increased risk of stroke. The beneficial effects of physical activity have also been documented for stroke. (30)

The protective effect of physical activity may be partly mediated through its role in reducing blood pressure and controlling other risk factors for cardiovascular disease, diabetes, and increased body weight. Other biological mechanisms have also been associated with physical activity, including reductions in plasma fibrinogen and platelet activity and elevations in plasma tissue plasminogen activator activity and HDL concentrations. (30)

## 2.4.4 PATOPHYSIOLOGY OF FOCAL CEREBRAL ISCHEMIA

### 2.4.4.1 Mechanisms of ischemic injury

Early observations on the mechanisms of ischemic injury focused on relatively simple biochemical and physiological changes which were known to result from interruption of circulation. Examples of these changes are: loss of high-energy compounds, acidosis due to anaerobic generation of lactate, and no reflow due to swelling of astrocytes with compression of brain capillaries. Subsequent research has shown the problem to be far more complex than was previously thought, involving the action and interaction of many factors. (15)

#### 2.4.4.2 Biochemical events

Within 20 seconds of interruption of blood flow to the mammalian brain under conditions of normothermia, the EEG disappears, probably as a result of the failure of high-energy metabolism. Within 5 minutes, high-energy phosphate levels have virtually disappeared (ATP depletion). (15) ATP is required for maintaining ionic gradients. The disruption of ionic gradients in neurons and glial cells is characterised by influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$ , efflux of  $\text{K}^+$  from the cells and cellular depolarization. Somatodendritic and presynaptic voltage-dependent  $\text{Ca}^{2+}$ -channels become activated and excitatory amino acids (eg. glutamate) are released to extracellular space, which further contributes to influx of  $\text{Ca}^{2+}$  into the cells directly through NMDA receptor and some AMPA receptors or indirectly through metabotropic glutamate receptors. Energy-dependent removal of excess glutamate from the extracellular space also becomes impaired. (17) Glutamate-mediated overactivation leads to opening of channels for monovalent ions resulting in influx of  $\text{Na}^+$  and  $\text{Cl}^-$ . Water follows passively contributing to cell swelling and development of edema. (3)

#### 2.4.4.3 Excitotoxins

A rapidly growing body of evidence indicates that excitatory neurotransmitters, which are released during ischemia, play an important role in the etiology of neuronal ischemic injury. The mechanisms by which excitotoxins cause cell injury is not yet fully understood. As already mentioned, it is known that they facilitate calcium entry into neurons. However, these agents are neurotoxic even in cell culture where the medium is calcium free. Those areas of the brain which show the most "selective vulnerability" to ischemia, such as the neocortex and hippocampus, are richly endowed with excitatory AMPA (alpha-amino-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-d-aspartate receptors). (15)

#### 2.4.4.4 Free radicals

Increase in intracellular  $\text{Ca}^{2+}$  causes mitochondrial  $\text{Ca}^{2+}$  overload leading to a leakage of mitochondrial membrane, cessation of already compromised ATP production, and burst of oxygen free radicals (42). Oxygen free radicals contribute to membrane lipid peroxidation, damage to DNA and proteins, and production of inflammatory mediators. (38)

#### 2.4.4.5 Lactic acidosis

While it is clearly not the sole or even the major source of injury in ischemia, lactic acidosis does apparently contribute to the pathophysiology of ischemia. It has been shown, for instance, that lactate levels above a threshold of 18 - 25 micromol/g result in currently irreversible neuronal injury. (15)

Decrease in pH as a consequence of lactic acidosis has been shown to injure and inactivate mitochondria. Lactic acid degradation of NADH (which is needed for ATP synthesis) may also interfere with adequate recovery of ATP levels post ischemically. Lactic acid can also increase iron decompartmentalization, thus increasing the amount of free-radical mediated injury. (15)

#### 2.4.4.6 Histological ultrastructural changes

Ischemic changes in cell architecture begin almost as rapidly as ischemic changes in biochemistry. Within seconds of the onset of cerebral ischemia, brain interstitial space almost completely disappears. Loss of interstitial space is a consequence of cell swelling secondary to sodium influx and failure of membrane ionic regulation. There have been several studies of the ultrastructural alterations associated with prolonged global cerebral ischemia in the rat. (15)

The hemodynamic, ionic and metabolic changes do not affect the ischemic territory homogenously. In the centre of the ischemic territory, core, the CBF is below 10% of normal. This area is destined to die and the cells are killed rapidly. Between the core

and normal brain tissue lies penumbra, an area with reduced CBF (20-60% of normal) containing functionally depressed but viable cells with maintained ionic homeostasis. If blood flow in this area further decreases below the critical level or energy demand is exceeded, the infarct area will expand. Ischemic penumbra is potentially salvageable by pharmacological intervention. (38)

#### 2.4.5 CLINICAL PRESENTATION

Regardless of the etiology, the clinical presentation of symptoms in a cerebral ischemic/infarction event depend upon:

- the location of the vessels
- the presence of collateral circulation.

However, embolic strokes have a greater risk of subsequent bleeding into the infarcted area than thrombotic strokes. (69)

##### Lacunar infarcts:

These are small cavity infarcts (usually <15 mm wide) are usually associated with hypertension. They can be either silent with no associated clinical symptoms or present with very obvious symptoms. The most common symptoms seen with lacunar infarcts are either pure motor deficits or pure sensory deficits. (55)

##### The middle cerebral artery:

Complete occlusion of the middle cerebral artery results in loss of movement and sensation on the contralateral or opposite side of the infarction. However, there may be a gaze preference to the ipsilateral or same side as the injury. If this is the categorical hemisphere, there is also a global aphasia. (69) If it is the representational hemisphere, there is a neglect syndrome, or forgetting that there are two sides of the body. Partial occlusion of the middle cerebral artery could include weakness of a hand or arm, facial weakness, and partial aphasia. (55)

##### The posterior cerebral artery:

There are 2 syndromes observed with occlusion of the posterior cerebral artery: P1 and P2. The P1 syndrome includes the midbrain, subthalamic, and thalamic regions of the brain. The symptoms include a third-nerve palsy with weakness and ataxia. Extensive infarction in this area can result in coma, unreactive pupils, and decerebrate rigidity. The P2 syndrome results in injury to the medial temporal and occipital lobes. This results in memory loss, disturbances, or loss of vision on the contralateral or opposite side of the infarct. In some individuals, visual disturbances can produce hallucinations.

The basilar artery:

The clinical presentation seen with occlusion of the basilar artery includes the “locked-in” state of preserved consciousness with total quadriplegia. A TIA in this area may produce dizziness or a feeling of light-headedness.

Superior cerebellar artery:

Occlusion of this vessel causes cerebellar ataxia, partial deafness, nausea, vomiting, and loss of pain and temperature sensation on the opposite extremities.

Anterior inferior cerebellar artery:

The symptoms seen with occlusion of this vessel include ipsilateral deafness, facial weakness, and vertigo. (69)

## 2.4.6 THERAPEUTIC STRATEGIES

Recovery from ischemic stroke is quite variable but typically follows a time course in which most improvement occurs over 1-6 months. To the extent that recovery occurs, function appears to be redistributed to parallel pathways or to new alternate pathways, or both. For example, partial recovery from the contralateral hemiparesis associated with a stroke involving the primary motor cortex sometimes involves activation of regions in the opposite, undamaged hemisphere. (73, 85, 22) In other cases, activation of regions ipsilateral to the stroke occurs as new neurons are recruited to perform specific tasks. (85, 73, 22) Similar shifts have been demonstrated during recovery in rodent models. (16)

Physicians have a wide range of therapies to choose from when determining a stroke patient's best therapeutic plan. The type of stroke therapy a patient should receive depends upon the stage of disease. Generally there are three treatment stages for stroke: prevention, therapy immediately after stroke, and post-stroke rehabilitation. (62)

#### 2.4.6.1 New strategies

Multiple novel strategies to reduce ischemic stroke burden are currently being investigated. It is expected that many of these strategies will be incorporated into our growing arsenal of stroke therapies and will allow us to reduce stroke-related mortality and morbidity. Multimodal combinations of novel lytic agents with mechanical reperfusion devices and stents and their further combinations with newer effective neuroprotectants may increase the therapeutic effects even further. Another approach to improve stroke outcome is to enhance late healing processes in the brain by cell and growth factor therapy. (44)

##### ***Prestroke***

- Prophylactic neuroprotection for high-risk patients?

##### ***Minutes to hours after stroke***

- Acute reperfusion therapies
- Intravenous thrombolysis
- Intra-arterial thrombolysis
- Combined intravenous/intra-arterial thrombolysis
- Mechanical reperfusion techniques

##### ***Minutes, hours, or days after stroke***

- Neuroprotective therapy: chemotherapy cocktail of agents targeting different aspects of the ischemic cascade, perhaps administered sequentially at various time points after stroke (Antinecrotic, Antiadhesion/anti-inflammatory agents, Antiapoptotic agents)
- Combined thrombolysis and neuroprotective therapy
- Tight control of glucose, perhaps insulin administration

- Tight control of temperature, perhaps antipyretic administration, perhaps hypothermia

***Days, weeks, or months after stroke***

- Restorative treatments targeting specific deficits, eg., gait retraining, arm function
- Pharmacotherapy coupled closely with rehabilitation (rehabilitation pharmacology)?
- Growth factors coupled with rehabilitation?
- Stem cell therapy coupled with rehabilitation?
- Gene therapy?

***Ongoing***

- Avoidance of "detrimental" drugs
- Secondary stroke prevention therapies, including combination antithrombotic agents

**Figure 2.** Possible Stroke Treatment Options for the Future: Evolving Time Windows and Combination Therapy Approaches (28)

**Cell-mediated strategies**

Recent studies have highlighted the enormous potential of cell transplantation therapy for stroke. A variety of cell types derived from humans have been tested in experimental stroke models. Cell transplantation therapy for stroke holds great promise. (6)

Cell-mediated strategies seek to partially restore the neural controls for complex cognitive, sensory, or motor functions. Cellular therapies, then, and perhaps other types of biologic and pharmacological interventions, could serve as adjuncts to the neurorehabilitation program of highly impaired subjects. Therapeutic cells may be capable of enhancing fundamental mechanisms for learning within circuits that they reconstruct or modulate, as well as by inducing, delivering, or extending in time peri-infarct and more distant molecular signals for neurogenesis, cell differentiation, axonal and dendritic sprouting, network connectivity, and long-term potentiation. These gene-induced, biochemical, physiological, and morphological modulations may be especially important for optimizing the function of spared pathways. (18)



White matter lesions may disrupt association, commissural, fascicular, and other projection fibers. If demyelination exists among spared axons, a remyelination strategy with stem cells or oligodendrocyte precursors to rescue axons becomes of interest. (18)

Cellular transplants could modulate the sprouting of spared axons and the regeneration of disrupted axons by providing neurotrophins or acting on axon growth cone inhibitors, such as Nogo-A. (68) Cells with or without gene modifications for producing trophic or tropic substances could be placed into the perilesional or distal milieu to augment axon growth through white matter. The complexity of long distance axonal regeneration is formidable, however. The architectural barriers and the balance of pro-growth and inhibitory molecules in the milieu must be managed to guide axons through white matter to their targets. Axon extension beyond the lesion site has been accomplished in select experimental conditions in animal models, however. (13, 87)

#### 2.4.6.2 Stroke rehabilitation

Rehabilitation plays a key role in improving outcome from stroke. Rehabilitation occurs in multiple physical domains. Scientific study is beginning to define the mechanisms that underlie neural and cortical recovery from stroke. (91)

Post stroke therapeutic interventions should be appropriately timed and directed toward the specific neural substrate to maximize cortical reorganization. It is recognized that the earlier the implementation of therapy, the better functional outcome. (67) A 2005 American Stroke Association endorsed Clinical Practice Guideline recommends that rehabilitation therapy starts as soon as possible. (19)

There are four primary areas of focus for therapeutic rehabilitative intervention in stroke patients: mobility, self-care, language deficits and swallowing impairment. The rehabilitation physician also plays a role in detecting and treating spasticity and mood disorders following stroke. (91)

The developing evidence- based methodologies that are becoming the foundation for stroke rehabilitation, will likely improve the efficacy and efficiency of rehabilitation

interventions. Through this process, stroke survivors will continue to experience better functional outcomes and ultimately, a better quality of life. (91)

### **3. AIMS AND HYPOTHESIS**

#### **3.1 AIMS**

The aim of this study is to characterize the regional patterns of neural and axonal injury following Endothelin-1(ET-1) induced focal cerebral ischemia in immature rats.

##### Description of the lesion:

- I. Assessment of gray matter damage and white matter damage (axons and oligodendrocytes) with use of conventional morphological techniques
- II. Assessment of the correspondence between white matter injury location and regional neurodegeneration.
- III. The injury will be assessed qualitatively.

#### **3.2 HYPOTHESIS:**

- I. We hypothesized that damage to both, neuronal perikarya and axons; occur following ET-1 induced focal cerebral ischemia.
- II. We assume that the disappearance of stained myelinated fibers and the appearance of pathological myelin structures corresponds well to the degeneration of neurons taking place following ET-1 induced focal cerebral ischemia.

## **4. METHODS**

### **4.1 ANIMALS AND SURGERY**

#### **4.1.1 CHARACTERIZATION OF THE GROUP OF ANIMALS**

Experiments were performed in Wistar albino rats on postnatal day 12 (P12; n= 9), or 25 (P25; n= 10). The day of birth was defined as day 0. Rats were housed in a controlled environment (temperature  $22 \pm 1^{\circ}\text{C}$ , humidity 50-60%, lights on 06:00-18:00 h) with free access to food and water. Experiments were approved by the Animal Care and Use Committee of the Institute of Physiology of the Academy of Sciences of the Czech Republic. Animal care and experimental procedures were conducted in accordance with the guidelines of the European Community Council directives 86/609/EEC.

#### **4.1.2 PRODUCTION OF FOCAL CEREBRAL ISCHEMIA**

Endothelin-1 is generally considered the most potent vasoconstrictor with a long/lasting action. Injection of ET-1 into the brain parenchyma has been found to induce substantial reduction of local blood flow followed by development of focal lesion. Recently focal injection of ET-1 was proposed as a highly reproducible model of human stroke. (92) The ET-1 model of focal cerebral ischemia incorporates reperfusion.

Surgery was performed under halothane anesthesia. Anesthesia was induced in an inductory chamber and then the animals were placed into a stereotaxic apparatus with a breathing mask adapted for neonatal rats for continuous delivery of anesthesia. Rat pups were maintained at 0.8 - 1.5% halothane throughout the rest of procedure. Cannula for Endothelin-1 infusion (PlasticsOne, Bilaney – Germany; outer diameter 0.2mm) was inserted into left hemisphere at coordinates AP= 0; L= 2; V= 2 in P12 and AP= 0; L= 2; V= 2.5 relative to bregma in P25 animals.

Endothelin-1 (#E7764, Sigma, St Louis, MO, U.S.A.) was dissolved in 0, 01 M phosphate buffered saline (PBS, pH 7, 4) and infused using an infusion pump (KDS210, KD Scienrific, Holliston, MA, U.S.A.; 1µl/5min) to freely moving animals. Controls received 0, 01 M PBS in corresponding volume instead of ET-1 solution (P12 n=, P25n=). The dose of ET-1 used in both age groups was 40 pmol. After the injection, the cannula was kept in place for 5 minutes to prevent capillary rising to the cannula track.

## **4.2. HISTOLOGY**

### **4.2.1 FIXATION**

All rats were deeply anesthetized with urethane (2.5 g/kg, i.p.) and transcardially perfused according to Timm-fixation protocol (one of the staining techniques that have been made on the slices) with 0.37% sulfide solution (1 ml/g) for 10 minutes followed by 4 % paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (1 ml/ g), +4<sup>0</sup>C, for 10 minutes. The brains were removed from the skull and postfixed in buffered 4% paraformaldehyde for 3 hours and then cryoprotected in gradual sucrose (10, 20 and 30%) at +4<sup>0</sup>C. Then the brains were frozen in dry ice and stored at -70<sup>0</sup>C.

The brains were sectioned in the coronal plane (50 µm, 1-in-5 series) with Cryocut Leica 1600 and sections were stored in a cryoprotectant tissue-collecting solution (30% ethylene glycol, 25% glycerol in 0.05 M sodium phosphate buffer) at -20<sup>0</sup>C until processed. Adjacent series of sections were used for Fluoro-Jade B and Black Gold staining.

### **4.2.2 FLUORO-JADE B STAINING**

To detect and localize degenerating neurons, adjacent series of sections were processed for Fluoro-Jade B. This fluorescent marker of neuronal degeneration was obtained from Histo-chem (USA).

The sections were mounted on 2% gelatin coated slides and then air dried on a slide warmer at 50°C for at least half an hour. The slides were first immersed in a solution containing 1% sodium hydroxide in 80% alcohol (20 ml of 5% NaOH added to 80 ml absolute alcohol) for 5 min. This was followed by 2 min in 70% alcohol and 2 min in distilled water. The slides were then transferred to a solution of 0,006% potassium permanganate for 10 min, preferably on a shaker table to insure consistent background suppression between sections. The slides were then rinsed in distilled water for 2 min.

The staining solution was prepared from a 0,01% stock solution of Fluoro-Jade B that was made by adding 10 mg of the dye powder to 100 ml of distilled water. To make up 100ml of staining solution, 4 ml of the stock solution was added to 96 ml of 0,1% acetic acid vehicle. This results in a final dye concentration of 0,0004%. The stock solution, when stored in the refrigerator was stable for months, whereas the staining solution was prepared within 10 min of use and was not reused. After 20 min in the staining solution, the slides were rinsed for one min in each of three distilled water washes. Excess waster was removed by briefly draining the slides vertically on a paper towel. The slides were then placed on a slide warmer, set at approximately 50°C, until they were fully dry (e.g. 5-10 min). The dry slides were cleared by immersion in xylen for at least a min before coverslipping with Canadian balsam, a non-aqueous, non-fluorescent plastic mounting media.

#### 4.2.3 BLACK-GOLD STAINING

A novel haloaurophosphate complex called Black-Gold has been synthesized and applied to localize myelin within the central nervous system. The technique is tailored to studies using formalin fixed non-solvent processed tissue. The technique stains large myelinated tracts dark red-brown, while the individual myelinated axons appear black. Black-Gold is a sensitive method for detection of pathology in excitotoxin-induced myelin morphology. (82)

This bright field myelin stain was obtained from Histo-chem (USA). Slide-mounted sections were rehydrated for 2 min in distilled water, and incubated for 15 min at 60°C in a solution of 0,2% Black-Gold dissolved in 0,9% saline. The sections were briefly

washed in distilled water and then intensified by incubation for 15 min at 60°C in 0,2% potassium tetrachloroaurate dissolved in 0,9% saline. Slides were rinsed in distilled water for 1 min, and then fixed in 2% sodium thiosulfate solution for 3 min. The slides were subsequently rinsed for 5 min in each of three changes of tap water. Sections were then dehydrated with graduated alcohol solutions and by air drying with a slide warmer. Dehydrated slides were then briefly cleared by immersion in xylene and coverslipped with Candian balsam.

#### **4.4 ANALYSIS**

In order to detect and localize degenerating neurons, each FJB-stained section throughout the brain was analyzed. Damage extension was evaluated separately in individual areas of the brain (e.g. the hippocampus, the neocortex).

To recognize the morphological features of the brain, each sample was compared to anatomical atlas of Paxinos and Watson. (70)

The tissue was examined using an epifluorescent microscope Olympus AX70 with high sensitive cooled 3CCD digital camera Olympus DP70.

Sections stained with Black-Gold were matched using anatomical landmarks (70) and inspected by light microscopy. As described before the technique stains large myelinated tracts dark red-brown, while the individual myelinated axons appear black. This novel tracer localizes both normal and pathological myelin.

Damaged areas were mapped onto coronal sections in the atlas of Paxinos and Watson. (70) For the microscopy, lenses with 2x, 10x, and 20x magnification were utilized.

The investigator who evaluated the extent of damage was blind to the status of the animals.

## **5. RESULTS**

### **5.1 GENERAL OBSERVATIONS**

Ischemic damage to neuronal cells and to myelin was observed in all animals with 40 pmol of ET-1. In contrast, the sections from vehicle treated animals revealed minimal neuronal cell body damage, limited to a small area at the injection site. ET-1 caused neuronal degeneration in a bigger area surrounding the injection site, indicated by the presence of FJB-positive cells. Image analyses performed on stained brain sections after ET-1 administration, revealed damage that encompassed cortical, alveolar, callosal and in some animals even hippocampal regions ipsilateral to the ET-1 injection.

Contralateral hemispheres revealed the typical normal pattern of labeling for each respective marker in both age groups of animals. Specifically, the FJB staining resulted in virtually no label within the brain itself. Occasionally a relatively small number of FJB-positive neurons were found in the contralateral hemisphere of both animal groups. This slight apparent damage varied from animal to animal and varied somewhat across medial to lateral extent of the brain and was possibly associated with mechanical effects of the injection, of craniotomy, or its impact on the brain removal process.

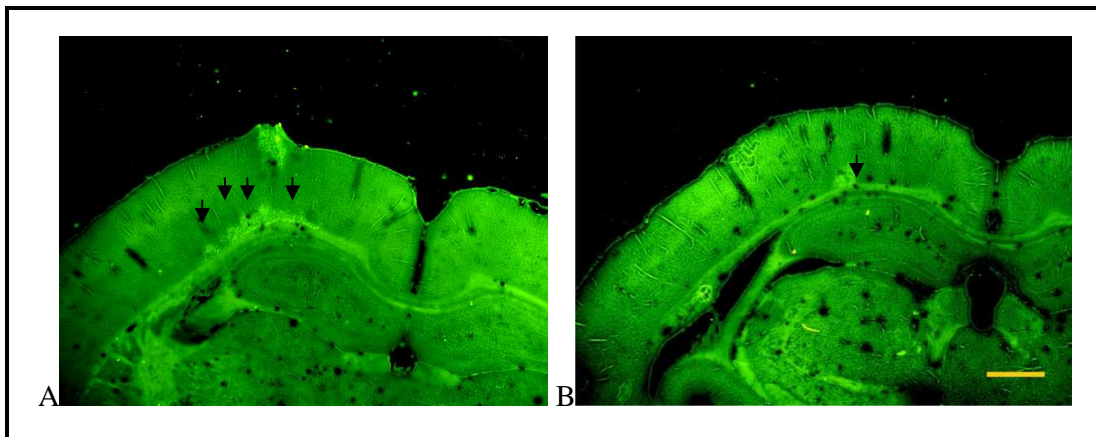
Black-Gold staining resulted in the conspicuous labeling of all myelinated fibers within all brain regions examined.

A constant feature in all animals was that the extent of the lesion seemed bigger in more coronal planes located more caudally from the injection site than rostrally from the injection site.



## 5.2 PATHOLOGICAL FEATURES OF AXONS, OLIGODENDROCYTES AND DAMAGED NEURONAL PERIKARYA IN THE CEREBRAL CORTEX

Neuronal damage was found in the cortex surrounding the injection site in both animal groups. The wide-spread of the area, where degenerating neurons were observed in the ET-1 treated animals was bigger than the area, where this type of damage was found in sham operated animals. Only sparse FJB-positive neurons were detected in the close vicinity of the cannula track and in the injection site in vehicle treated animals.



**Figure 3.** Intensely Fluoro-Jade B labeled cells (indicated with arrows) are observed in the penumbra immediately adjacent to the injection site in the ipsilateral cortex of the ET-1 treated animals (A) and in the animals from the control group (B). The wide-spread of Fluoro-Jade positive cells is larger in ET-1 treated animals (A) as compared to animals from the control group (B). Scale bar= 500  $\mu$ m.

Pathologic features of myelin and myelin loss was observed in the cortical region adjacent to the injection site in both, ET-1 and sham group. The wide-spread of damage to myelin seems bigger in the ET-1 group as compared to the control group. An overt loss of myelinated fibers was observed in the ipsilateral cortex as compared to the contralateral cortex in both groups. Myelinated fibers in the cortex immediately

adjacent to the cannula track and the injection site were not apparent, but with growing distance from the cannula track and injection site, myelin fibers gradually appeared. A very moderate level of myelin damage was apparent in sham stroke control animals within a similar region to animals, which underwent the application of the ET-1 injection. (See att. 2-3)

In the lower layers of the cortex of the ET-1 treated animals a typical pattern of injury to myelin was observed in the coronal sections located caudally from the coronal section, where the injection site is detected.

In the cortical layer VI:

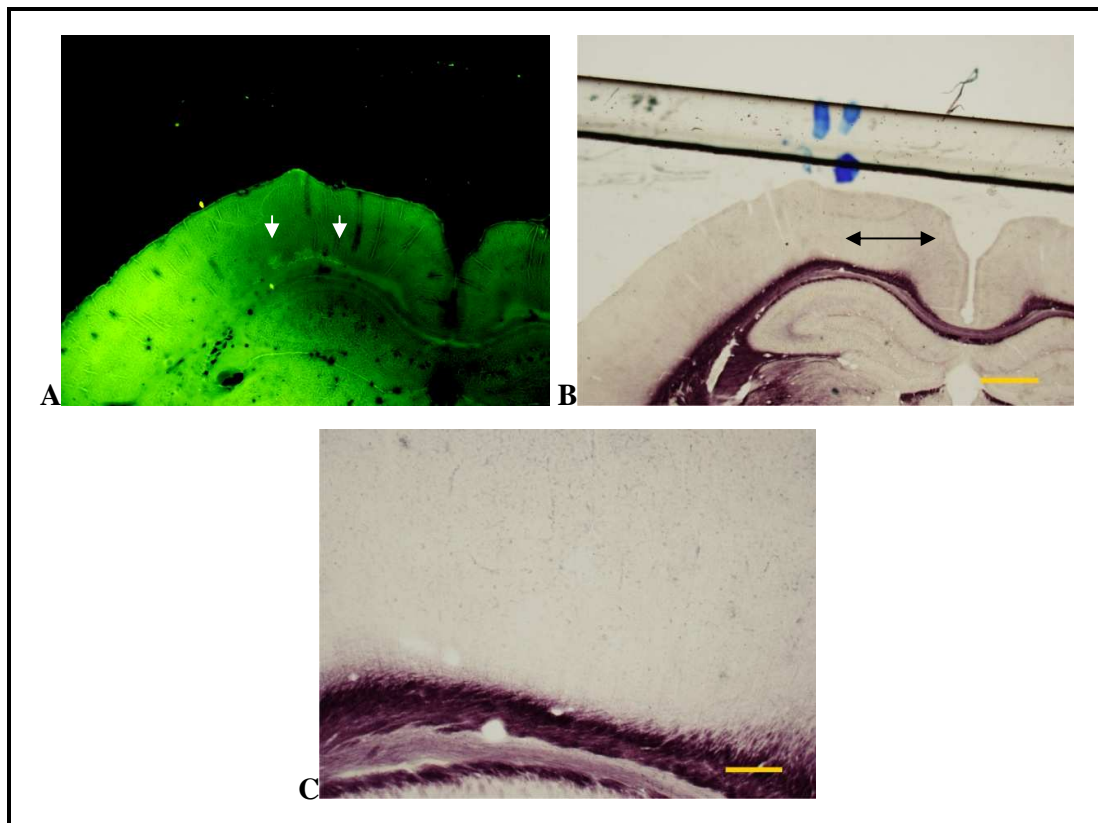
- Myelinated fibers appeared shorter, more divergent and their density decreased relative to sham and to the contralateral hemisphere of the same animal.
- The density of myelinated fibers of the cell layer VIa), where more vertically oriented pyramidal cells are located, decreased more than in the VIb) layer, where cells are oriented more horizontally.
- Observation of the myelin in layer VI within the ipsilateral cortex to the ET-1 injection revealed bead-like myelin structures. They were located well beyond the surface, where FJB-positive cells were found. (see att. 4)

In cortical layer V:

- The extent of ischemic damage was also of interest in this layer as cortical neurons in this layer are the major output neurons of the cortex that synapses with the midbrain, hindbrain and spinal cord, ultimately affecting motor function.
- In the sections immediately following the injection site, the disappearance of myelinated fibers in this layer was observed, suggesting that there are no fibers leaving, coming to or passing through this layer. (see att. 5)

Our observations of the layers V and VI revealed that the spatial spread of the ischemic damage to the white matter extended further laterally than the spread of FJB-positive cells.

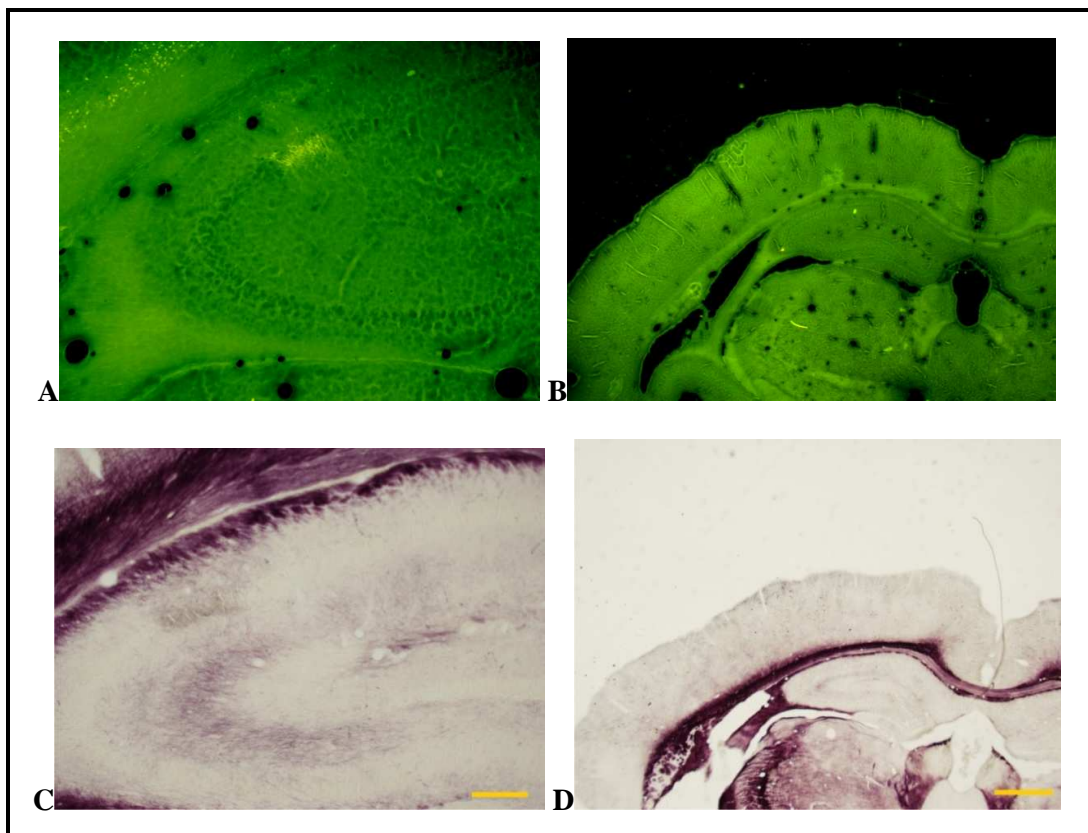
Although the size of the ischemic lesion decreased the further the section was located from the injection site, the lateral spread of damage to neuron cells remained relatively confined to the localization of the injection site in all animals. However, the damage to myelin seems to spread more septally in more caudal coronal planes following after the section, in which the injection site is detected.



**Figure 5.** Fluoro-Jade B staining and Black-Gold staining of an ET-1 treated animal coronal section located further caudally from the injection site (A-C). Fluoro-Jade staining reveals the presence of degenerating neurons in the ipsilateral cortex (white arrows indicate the wide-spread of degenerating neurons) (A). An adjacent Black-Gold labeled section demonstrates, that the localization of damage to myelin (black arrow) in the ipsilateral cortex is located more septally (B, C) in comparison to the localization of the degenerating neurons. Scale bar (A, B) = 500  $\mu$ m, scale bar (C) = 100  $\mu$ m.

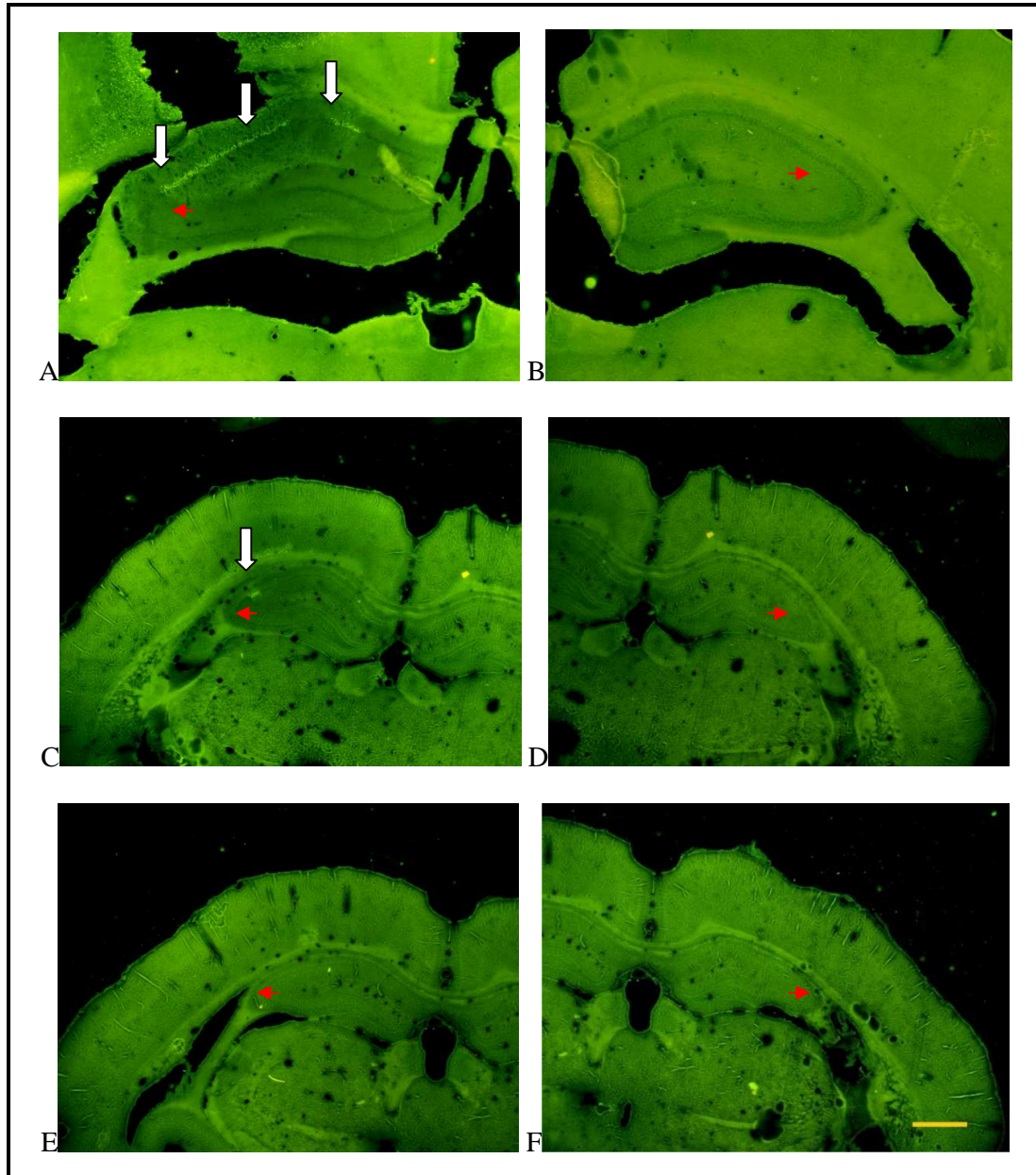
### 5.3 PATHOLOGICAL FEATURES OF AXONS, OLIGODENDROCYTES AND DAMAGED NEURONAL PERIKARYA IN THE HIPPOCAMPUS

Hippocampal injury was evident in 5 (P12=2, P25=3) animals from the ET-1 group in both Black-Gold and FJB staining. Damage was located in the dorsal part of the ipsilateral hippocampus. In controls, no FJB-positive neurons and no myelin pathology was detected neither in the ipsilateral nor in the contralateral HC.



**Figure 6.** Distribution of FJB– positive cells in the ipsilateral hippocampus of an ET-1 treated animal (A). The ipsilateral hippocampus of a control animal is free of FJB– positive cells (B). Black-Gold staining reveals damage to myelin in the ipsilateral hippocampus of an ET-1 treated animal (C). In the ipsilateral hippocampus no myelin pathology is observed (D). Scale bar (A, C) = 100 μm, scale bar (B, D) = 500 μm.

FJB labeled cells were most consistently noted in the ipsilateral stratum pyramidale and stratum radiatum of the CA1/2 border and CA1 area of the HC, whereas the contralateral HC is unlabeled. The CA3 area of the HC in both age groups was free of FJB-positive cells.



**Figure 7.** Fluoro-Jade B labeling in the ipsilateral (A, C, E) versus the contralateral (B, D, F) cortex in both, in a P12 ET-1 treated animal (A, B) and in a P25 treated animal (C, D), and in an animal from the control group (E, F). Fluoro-Jade B positive cells are

observed throughout the ipsilateral hippocampus including the cells in stratum pyramidale, stratum radiatum and stratum oriens of the border of the CA1 and CA2 area of the hippocampus and in the CA1 area of the hippocampus (white arrows), whereas the contralateral hippocampus is unlabeled (B, D, F). The CA3 area of the hippocampus is free of Fluoro-Jade B positive cells (red arrows) in the ipsilateral and contralateral hippocampus (A-F). Scale bar= 500  $\mu$ m.

The distribution of FJB-positive cells in the CA1 area of the HC of the ET-1 treated animals exhibited a specific „patchy pattern“, described by G. Tsenov et al. (see att.6)

The investigation of the presence of myelinated fibers in the HC subregions in the control groups revealed that most of the CA3 projections projected septally. In contrast, in the CA1 region, the overwhelming majority of myelinated fibers projecting from this hippocampal subfield were found in the stratum radiatum. Most myelinated fibers in the CA1 region were emitted caudally. (See att. 7)

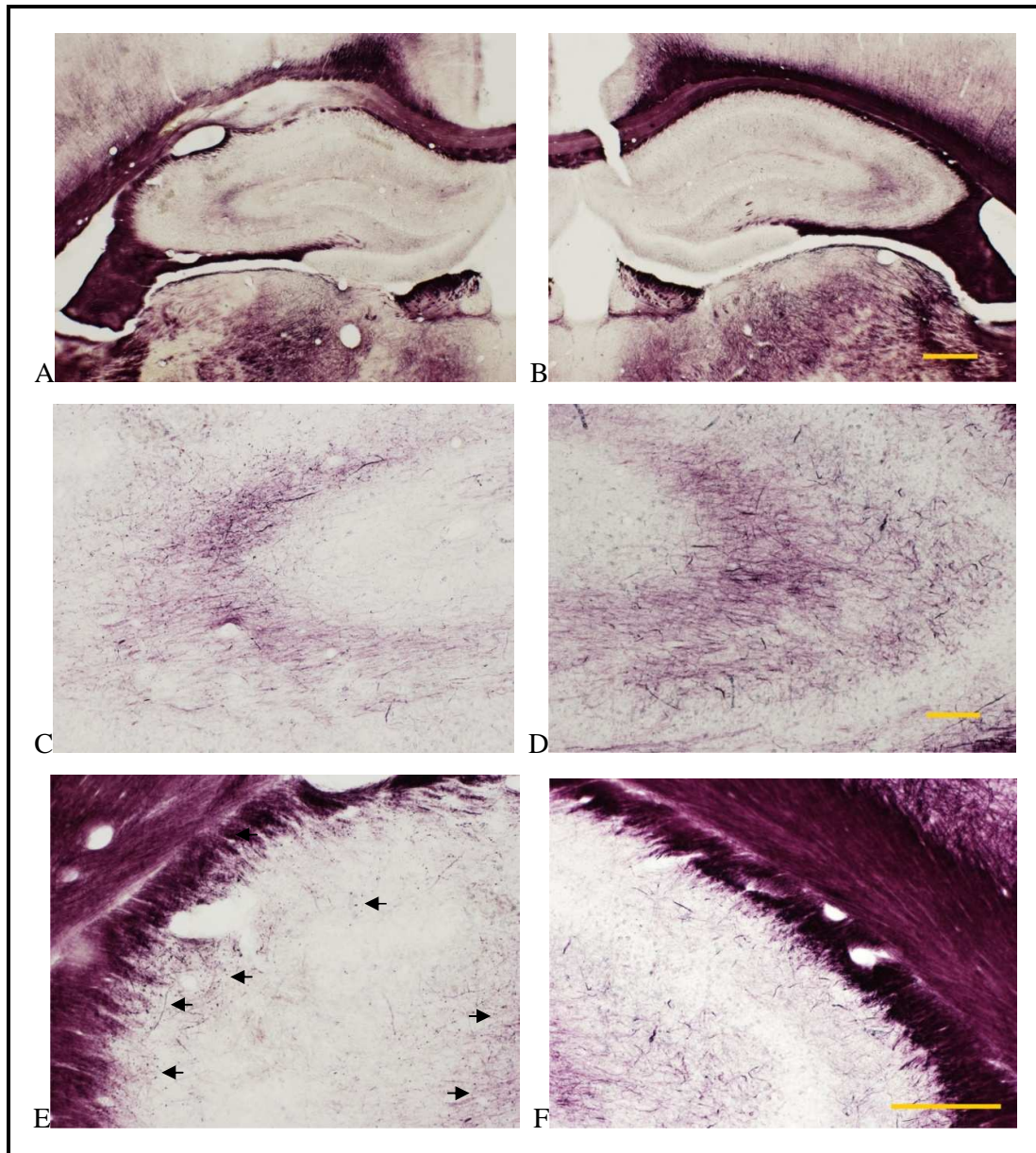
Differences between the sham and the ET-1 group were detected in the intensity of Black-Gold staining in the inspected subregion of the HC. In the control group, there was no evidence of myelin loss.

Myelin loss was observed in the ipsilateral stratum oriens et radiatum of the hippocampal CA1/2 border and CA1 in all the 5 mentioned animals from the ET-1 group, as compared to the contralateral hemisphere of these animals. This myelin loss was located within a similar region in which FJB-positive cells were detected in these animals. (See att. 8)

Despite the fact that myelinated fibers disappeared in the CA1 area of the hippocampus in all the 5 mentioned ET-1 treated animals, pathological myelin structures were apparent in the CA3 hippocampal subregion of the two P12.

Black-Gold staining revealed lesion-induced myelin pathologies in this region. Labeling of this region is more subtle in comparison to the contralateral hippocampus. Varicosities, tumescences and bead-like myelin structures are present in the affected zone. The myelinated fibers seem to be shorter, more divergent and show more complex branching patterns.





**Figure 8.** Lesion-induced myelin pathologies in the ipsilateral CA3 hippocampal area of a P12 ET-1 treated animal (A, C, E) versus the contralateral CA3 hippocampal area (B, D, F). Black-Gold labeling is more subtle in the stratum radiatum of the CA3 hippocampal area and the Black-Gold positive fibers seem to be shorter and more divergent (C) in comparison to the contralateral CA 3 region (D). The arrows indicate bead-like structures of pathological myelin in the fimbria of the fornix and in the stratum radiatum of the CA3 area of the ipsilateral hippocampus (E) as compared to the contralateral hippocampus (F). Scale bar (A, B) = 500  $\mu$ m, scale bar (C-F) = 100  $\mu$ m.





The hippocampus appeared to be more extensively injured in P12 than in P25 ET- 1 treated animals as demonstrated by a bigger wide-spread of FJB labeled structures and by the disappearance of myelin and/or the presence of pathological myelin structures in the cornu ammonis and hilar region.

## **6. DISCUSSION**

### **6.1. DISCUSSION- THE RESULTS**

There is a substantial need to understand the vulnerability of the developing brain to focal cerebral ischemia. In this study we describe and characterize the regional patterns of neuronal injury and axonal degeneration after ET-1 induced focal cerebral ischemia in immature rats at postnatal day 12 and 25.

#### **6.1.1 DAMAGE TO WHITE AND GRAY MATTER IN THE IPSILATERAL CEREBRAL CORTEX**

Our findings demonstrate that ET-1 induced focal cerebral ischemia in the developing brain generates neuronal and myelin loss in the ipsilateral cerebral cortex of both age groups of immature rats. It also causes a distinct pattern of subcortical neuronal injury and the wide-spread of white matter damage.

The results suggest that the cerebral white matter is highly vulnerable to the effects of focal cerebral ischemia. Pathological structures of myelin and the loss of myelinated fibers in the cerebral cortex seem to be concomitant with the presence of degenerating neurons. However, it seems that the spatial spread of damage to myelin extends further laterally throughout the cortex than does damage to neuronal cells.

Analyses of areas of pathology in the cerebral cortex in coronal sections revealed that at more caudal coronal planes the location of damage to myelin appears more septally than in more rostral coronal planes. This observation contrasted strikingly with the finding in control animals where damage to white matter remained confined to the location of gray matter damage. This septall spread of damage to myelin observed on caudal coronal planes could be due to the course and ramification of the vasoconstricted vessel.

The pathogenesis of demyelination after ischemia may be due to loss of mature oligodendrocytes or may be secondary to other processes such as microglial activation or loss of trofic support after axonal degeneration (32).

Many studies show that oligodendrocytes are vulnerable to a variety of hypoxic insults. Cerebral ischemia is known to cause a variety of changes as energy failure owing to the lack of oxygen and glucose, excitotoxicity and free radical formation that include nitric oxid and TNF- $\alpha$  production, all of which are toxic to oligodendrocytes. (83)

Our data suggest that the white and gray matter damage is not the same in different cortical layers. Layer VI and especially layer V seems to be more vulnerable to ischemia than the other cortical layers.

It has previously been established that certain subsets of neurons, including layer V neurons, are particularly vulnerable to ischemic damage. (21)

Layer V contains large, burst-firing pyramidal cells with long apical dendrites. These large cells project, depending on cortical area, to subcortical regions such as the superior colliculus and the pons. (89)

An explanation for the vulnerability of the cells in the cortical layer V is that the higher surface area to volume ratio of axonal processes makes them not able to buffer aberrant ion influxes as efficiently as other neuronal cell populations, thus making them more susceptible to ischemia/excitotoxicity. (21)

Furthermore the damage to gray and white matter in this layer could be associated with the degenerating cells in the VI layer. Local axon collaterals of these cells arborize primarily in layer V and VI in  $S_1$  and the callosum. (89)

It is also possible that processes in deeper cortical regions than layer V are less ischemic because of redundant horizontally oriented blood flow derived from adjacent territories that are less ischemic. (21)

### 6.1.1 DAMAGE TO WHITE AND GRAY MATTER IN THE IPSILATERAL HIPPOCAMPUS

White and gray matter damage in 5 ET-1 treated animals was detected in the ipsilateral subcortical white matter with the involvement of the alveus, corpus callosum and the hippocampus. Degeneration of the subcortical white matter could be due to the loss of cortico-cortical innervations following loss of neurons in the ipsilateral cortex. (90)

This degeneration could also be the result of the contusive effect of the craniotomy or of the mechanical effects of the injection or of the brain removal process. The diffusion of released cytotoxic factors such as glutamate, nitric oxide, or other factors from the dying core could lead to the mentioned degeneration. (98)

To detect degenerating neuronal cells we used Fluoro Jade B (81), which is capable to label dying neurons in a wide variety of contexts, regardless of the mechanism of cell death. Fluoro Jade B-positive cells were observed only in the CA1 area of the ipsilateral hippocampus and are located "under" the ischemic core in the cortex.

Following ET-1 induced focal cerebral ischemia, a specific degeneration takes place in the hippocampus, accompanied by the loss of myelinated fibers and the appearance of pathological myelin structures. In the CA1 hippocampal area the loss of myelin was visible in the adjacent coronal sections to those, in which degenerating neurons were observed.

Pathological features of myelin and the loss of myelinated fibers was observed in the CA3 hippocampal area of all the P12 ET-1 treated animals, which exhibited white matter damage in subcortical regions. No Fluoro Jade B-positive cells were found in this hippocampal region in the same animals. The absence of Fluoro Jade B-positive cells in the CA3 hippocampal region of these animals could be due to their distance from the injection site that is too big for the diffusion of cytotoxic factors to lead to degeneration of cells in this hippocampal region. Subtle Black-Gold labeling was observed in the ipsilateral hilus in all ET-1 treated animals, which exhibited white matter damage in subcortical regions. No Fluoro Jade B-positive cells were found in this hippocampal region in the same animals. The absence of Fluoro Jade B-positive cells in this region of these animals could also be due to their big distance from the injection site.

The presence of myelin pathological structures and the loss of myelinated fibers in the hilus, CA1 and CA3 area of the ipsilateral hippocampus could be linked. Adult CA pyramidal cells have been shown to project not only to CA 1 area in the Schaffer collateral pathway, but also toward the hilus of the dentate gyrus.

The damage in the CA1 hippocampal area to gray and white matter was observed in the coronal section, which showed the injection site, at the CA1/CA2 boarder of these

hippocampal areas. However, in more caudal coronal planes, the damage to white matter was confined only to the CA1 area of the hippocampus. We could speculate, that the mentioned observed spatial pattern of ischemic damage in the CA1 hippocampal area is related with the damage to the white matter of the CA3 hippocampal area.

As mentioned, all portions of CA3 and CA 2 project to CA1 but the distribution of terminations in CA1 depends on the transverse location of the CA3/CA2 cells of origin. CA3 cells located close to the dentate gyrus (proximal CA3), while projecting both septally and temporally for substantial distances, tend to project to levels of CA1 located septal to their location. CA3 cells located closer to CA1, in contrast, project more heavily to levels of CA1 located temporally. At, or close to, the septotemporal level of the cells origin, those cells located proximally in CA3 give rise to collaterals that tend to terminate superficially in the stratum radiatum with a clear preference for the more distal portions of CA1 (near the subicular boarder). Conversely, cells of origin located more distally in CA3 give rise to projection that terminate deeper in the stratum radiatum and the stratum oriens, with a preference for portions of CA1 located closer to the CA2 boarder.(1)

A key question is whether the developing brain exhibits a degree of vulnerability different from that of the adult brain after focal cerebral ischemia. There is evidence for age-related differences in the presence of myelinated fibers in the developing hippocampus. (54, 29, 98) These differences may influence the extent of damage to white matter in animals of different ages.

Although entorhinal fibers first reach the dentate gyrus at embryonic day 19, the perforant path is almost formed at postnatal day 5. Light and ultrastructural analyses revealed that mature fibers in the hippocampus occur in the second postnatal week. During postnatal development, increasing numbers of myelinated fibers appear and the distribution of myelinated fibers at postnatal day 25 is similar to that found in the adult. (54)

During early postnatal life, there is an exuberant outgrowth of local CA3 recurrent axons, and with maturation these recurrent collaterals are remodeled. The number of

axon branches decreases by 50% in adulthood in comparison with the second postnatal week. (29)

The extension of the damage in the ipsilateral hippocampus of P12 ET-1 treated animals is higher than in P25 treated animals. This observation could be the consequence of the mentioned alterations of the degree of myelination in animals of different ages. Studies demonstrated that ET receptors are over expressed during early postnatal development; therefore ET-1 might be more effective in P12 than in P25 rats. (92)

## **6.2. DISCUSSION- THE METHODOLOGY**

Some errors might appear in our conclusions. The protocol of the surgical procedure is relatively free from major problems. Care must be taken, when implanting the cannula for ET- 1 infusion at the right coordinates. Since the animals are very young, manipulation with their body must done with big care in order to prevent mechanical insults to the brain tissue. The errors in conclusions arise mostly from the histological procedure. During fixation, cryoprotection and freezing brain tissue shrinks and thus influences the observations. This could be neglected in the case that the tissue from the control and experimental groups shrink similarly. (40)

We assessed neuronal and axonal injury by means of qualitative morphologic methods. In this study we used the myelin-specific marker Black-Gold to localize normal and pathological myelin (82) and Fluoro Jade B, an anionic fluorescein derivate, to stain neurons undergoing degeneratinion (81).

Studies of myelin in the CNS based on histochemical techniques have to be interpreted with some caution, as results may be influenced by the following factors: signal-to-noise ratio, considerable variability in staining, staining of unknown molecules and complex multi-step prestainig procedures. (54)

Based on the fact, that Black-Gold is a gold chloride dye, one may speculate on the possibility that Black-Gold binds to other for the present unknown molecules. This could lead to falsely negative findings. And thus the brain sections of all animals were

Black-Gold stained in two Black-Gold 0,2% staining solutions in order to prevent variability in staining due to the exhaustion of the staining solutions.

## **7. CONCLUSIONS**

Collectively, our observations support the hypothesis that damage to neuronal cells and myelinated axons occurs in the rat brain following Endothelin-1 induced focal cerebral ischemia. Our data emphasize the profound effect that focal cerebral ischemia has on both gray and white matter in the immature rat brain.

Our results strongly suggest that pathological changes in myelinated fibers appear to be concomitant with neuronal injury. However, the spatial spread of the damage to myelinated fibers seems to be bigger than is the extent of the area, where degenerating neurons were detected.

What triggers the damage to myelinated fibers in our model of focal cerebral ischemia remains to be elucidated. Our findings did not establish a cause-and-effect relationship between the white matter damage and gray matter damage. To investigate such functional outcome, further experiments would be of interest.



## **8. ABBREVIATIONS**

AMPA -  $\alpha$ -amino-3-hydroxy-5- methylisoxazole-4-propionic acid

ATP - Adenosine triphosphate

CA1-4 - Region 1-4 of the Cornu Amonnis

CBF - Cerebral Blood Flow

CNS - Central Nervous System

CNTF - Ciliary neurotrophic factor

DNA - Deoxyribonucleic acid

DG - Dentate Gyrus

EC - Entorhinal Cortex

EEG - Electroencephalography

ET-1- Endothelin-1

FJB - Fluoro Jade B

IGF-1- Insulin-like Growth Factor 1

FCI - Focal Cerebral Ischemia

FGF-2 - Fibroblast Growth Factor 2

HC - Hippocampus

HDL- High Density Lipoprotein

MAG - Myelin-Associated Glycoprotein

MBP - Myelin Basic Protein

NMDA - N-methyl-D-aspartate

NT-3 - Neutrophin 3

OCs - Oligodendrocytes

OMgp - Oligodendrocyte-Myelin Glycoprotein

OPCs - Oligodendrocyte Precursor Cells

PDGF-A - Platelet Derived Growth Factor

PLP- Proteolipid Proteins

PNS - Peripheral Nervous System

TIA - Transient Ischemic Attack

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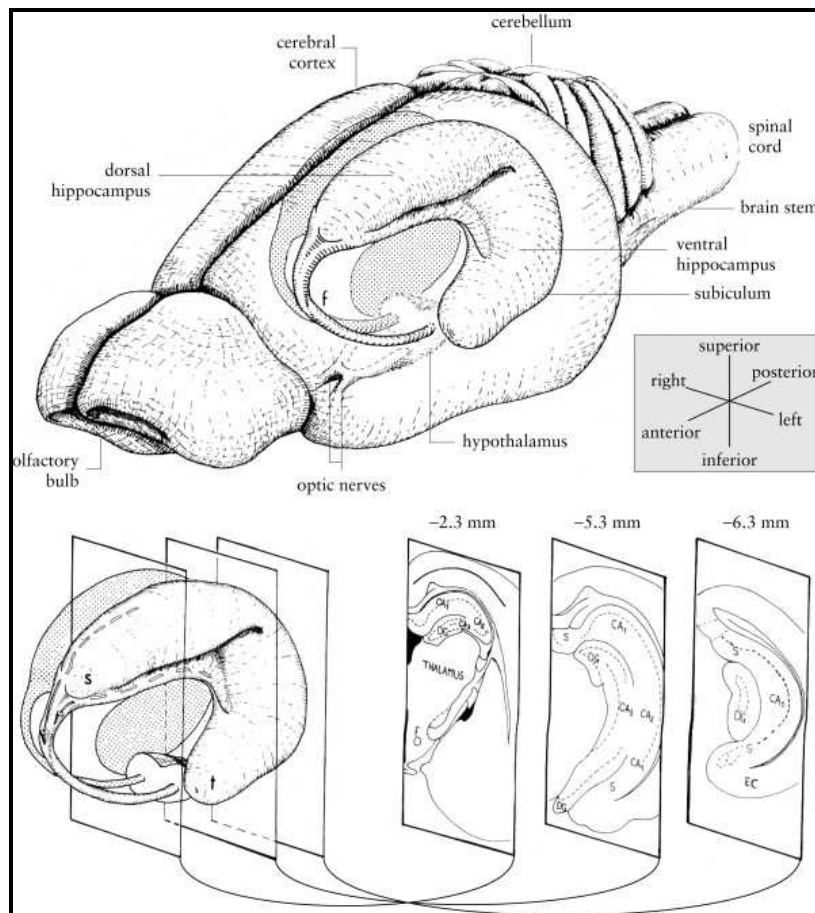
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## 10. ATTACHMENTS

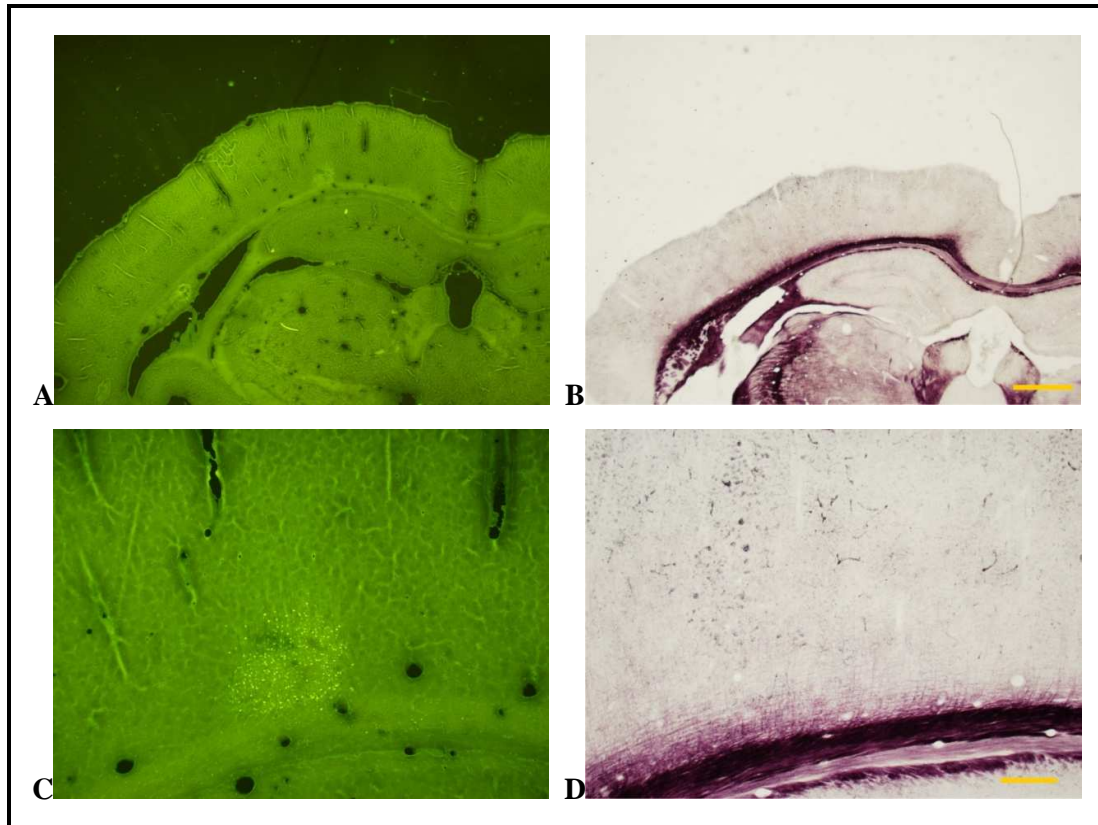
### Attachment No. 1



**Diagram of the rat hippocampus.** Drawings of the rat brain showing the three-dimensional organization of the hippocampus and related structures. Three coronal sections through the left hippocampus are shown at the bottom right of the figure, with their approximate anteroposterior coordinate relative to bregma. CA1, CA2, CA3: cornu

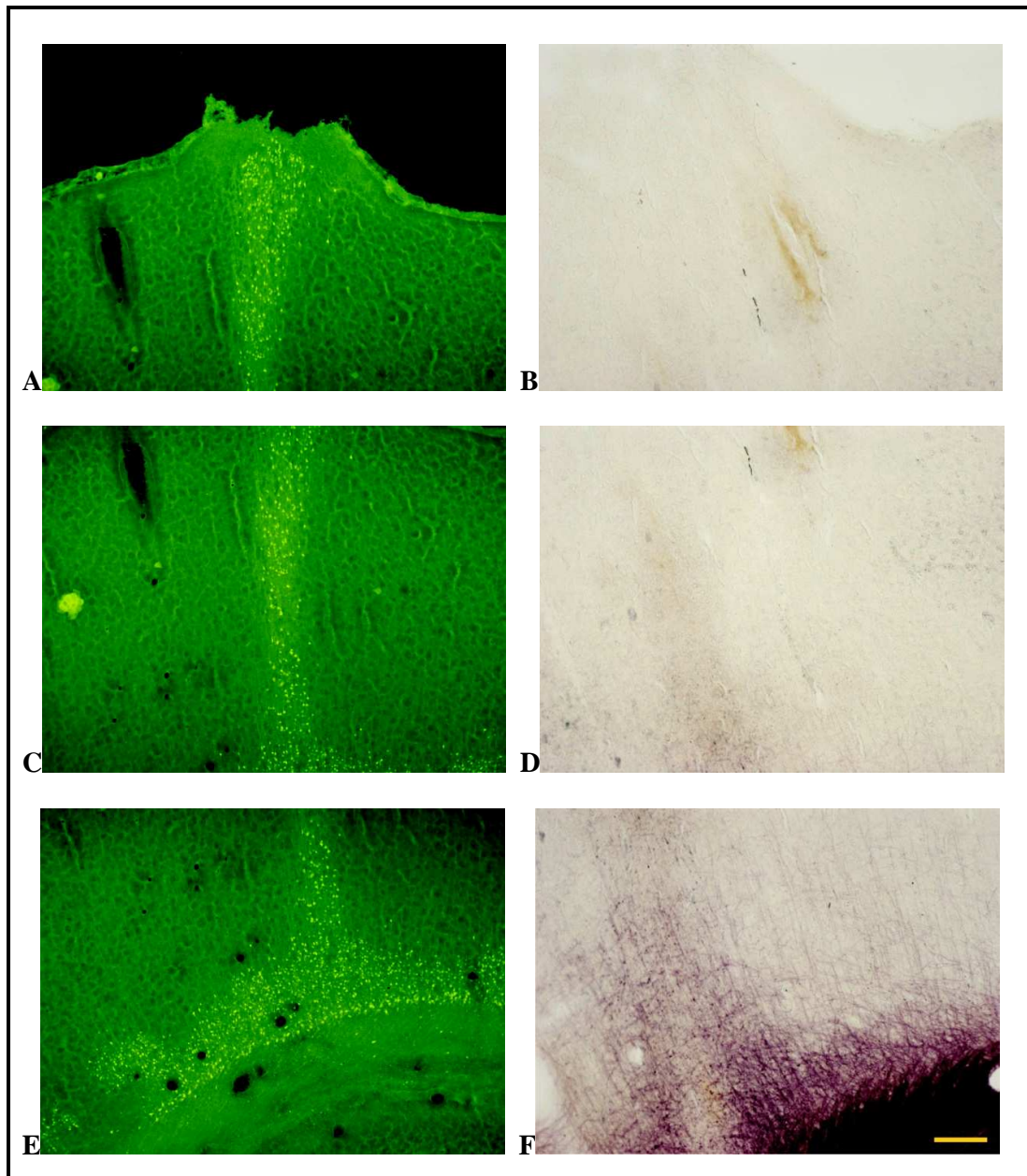
ammonis fields 1–3; DG: dentate gyrus; EC: entorhinal cortex; f: fornix; s: septal pole of the hippocampus; S: subiculum; t: temporal pole of the hippocampus. (14)

## Attachment No. 2



Fluoro-Jade B labeling (A, C) versus Black-Gold labeling (B, D) in the ipsilateral cortex in an animal from the control group. Fluoro-Jade positive neural profiles and the disappearance of myelin are observed in the close vicinity of the cannula track and in the close vicinity of the injection site. Scale bar (A, B) = 500  $\mu\text{m}$ , scale bar (C, D) = 100  $\mu\text{m}$ .

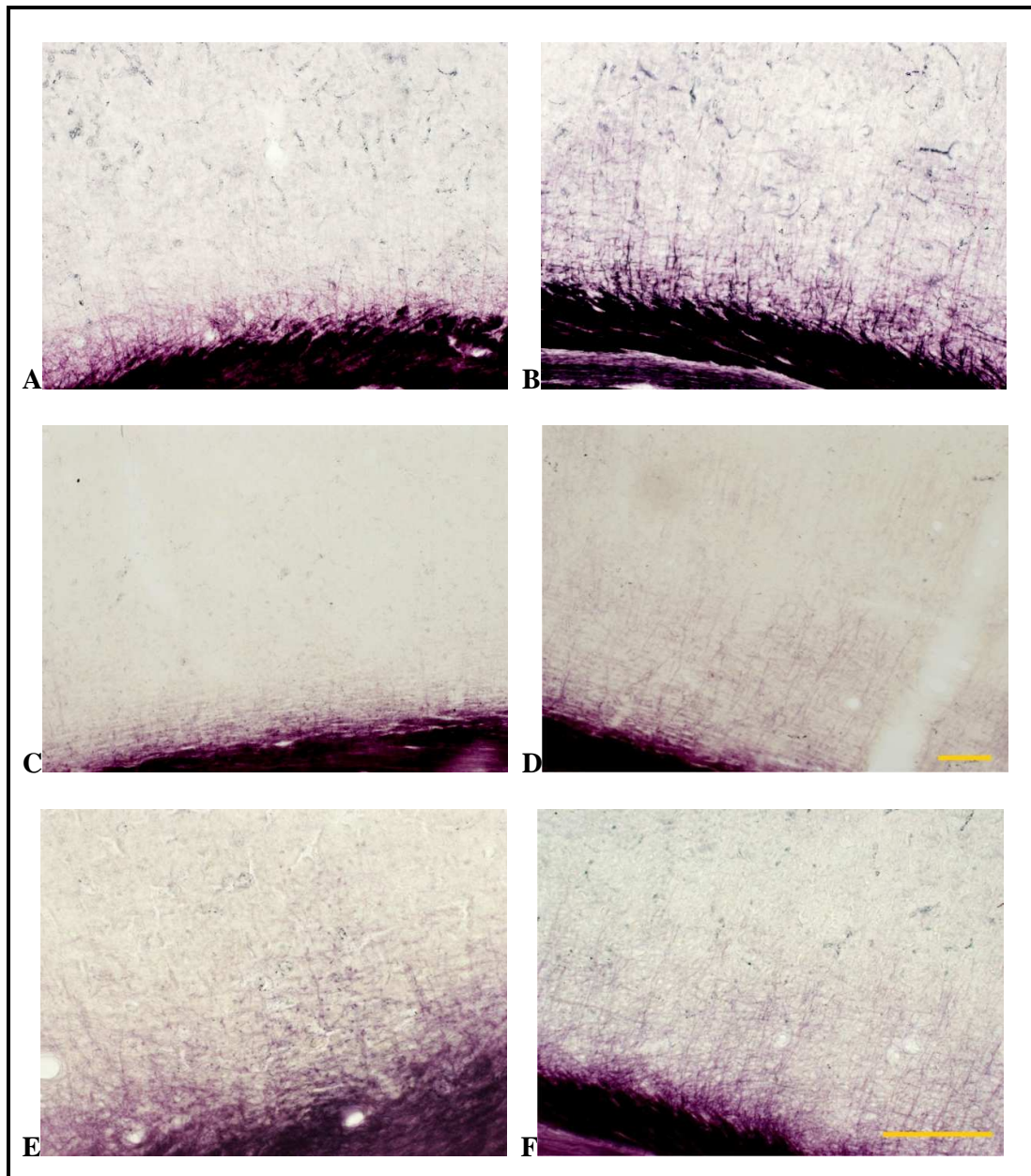
### Attachment No. 3



Prominent Fluoro-Jade B labeling is apparent in the ipsilateral cortex surrounding the cannula track and the injection site. (A, C, E) in the cortex of an ET-1 treated animal. Adjacent Black-Gold sections reveal white matter degeneration in similar regions (B, D, and F) in the same animal. Scale bar= 100  $\mu$ m.



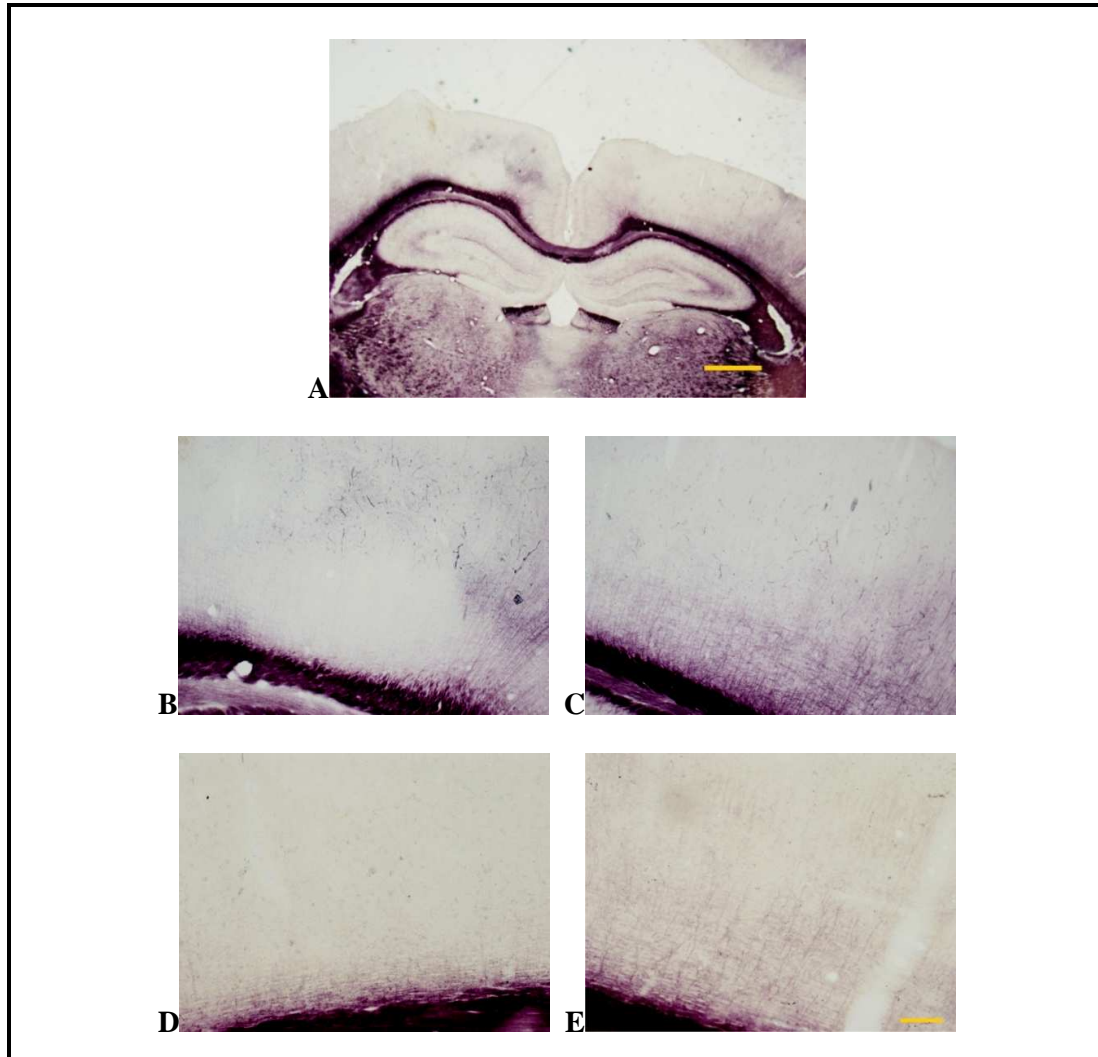
#### Attachment No. 4



Black-Gold staining reveals changes in the presence of myelin in the VI layer of the cortex in coronal sections of ET-1 treated animals (A- E). Black-Gold positive fibers in the ipsilateral cortex surrounding the injection site appeared shorter, more divergent and their density decreased (A) relative to the contralateral hemisphere (B). In the ipsilateral

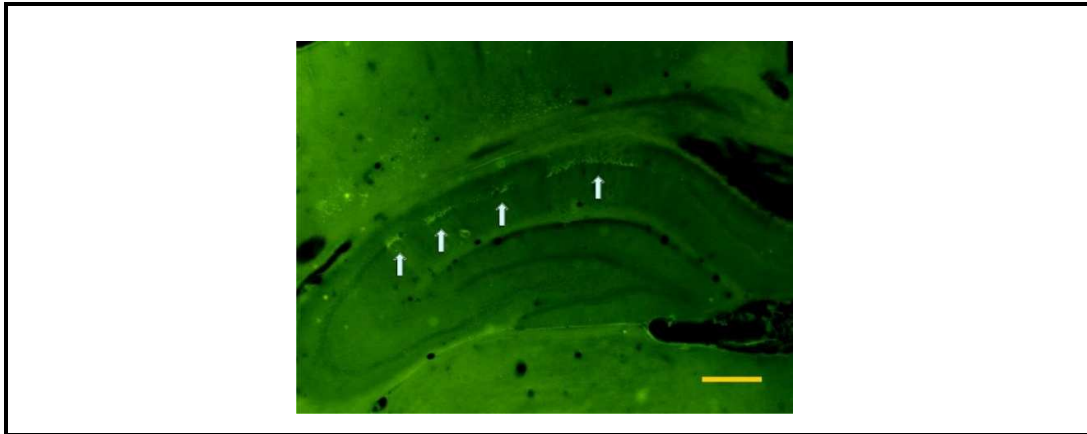
cortex mildly labeled fibers are visualized in the white matter of the VI a) cell layer and somewhat weaker staining is detected in the VI b) Layer (C), whereas Black-Gold positive fibers are present in both subunits of the VI cortical layer in the matching region of the contra lateral cortex (D). Bead-like myelin structures are present in the VI layer of the ipsilateral cortex surrounding the injection site (E). The contralateral cortex is devoid of bead-like myelin structures (F). Scale bar (A-D) = 100  $\mu\text{m}$ , Scale bar (E, F) = 100  $\mu\text{m}$ .

## Attachment No. 5



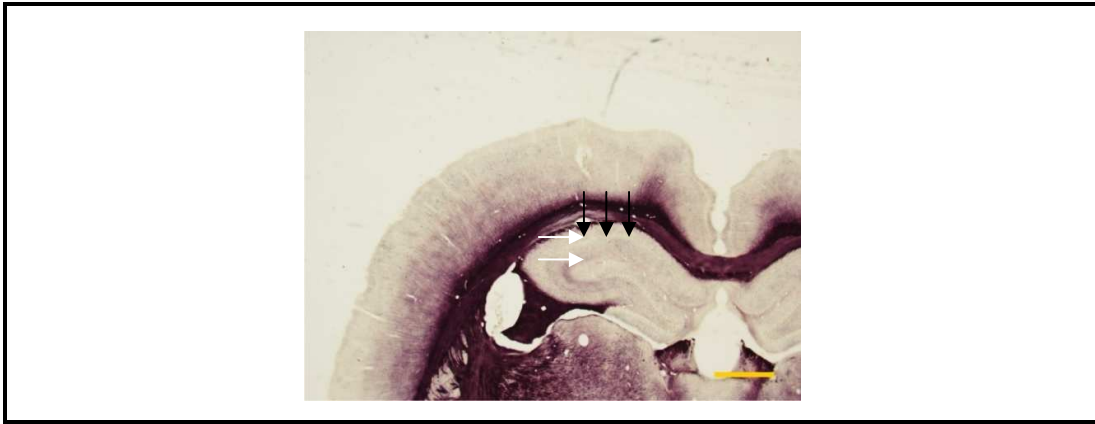
Black-Gold staining of an ET-1 treated P25 rat (A-C) and of an ET-1 treated P12 rat. The V. layer of the ipsilateral cortex of ET-1 treated animals (A, B, C), adjacent to the injection site, appears devoid of Black-Gold stained fibers relative to the contralateral cortex (A, C, E). Scale bar (A) = 500  $\mu\text{m}$ , scale bar (B-E) = 100  $\mu\text{m}$ .

## Attachment No. 6



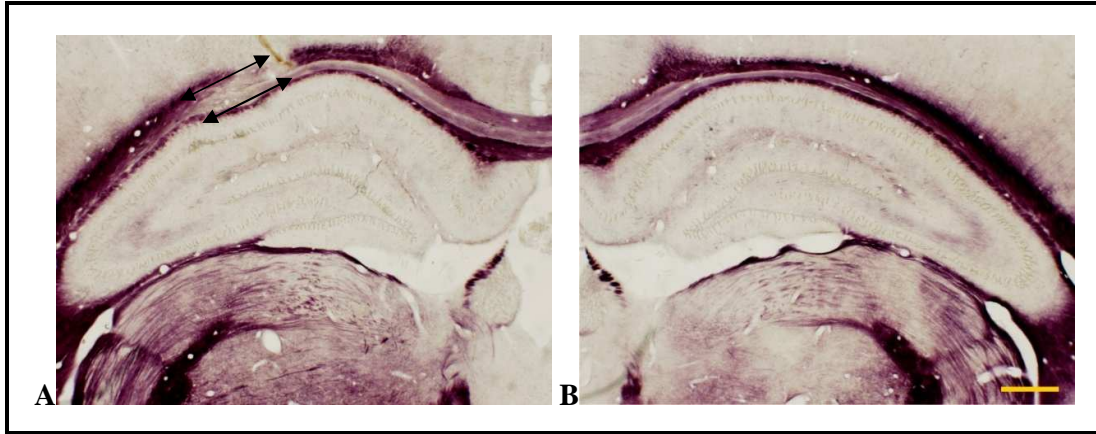
The distribution of Fluoro Jade B-positive cells in a P12 animal after infusion of ET-1. Fluoro Jade B-positive neurons (indicated by arrows) in the pyramidal cell layer of CA1. Scale bar= 500  $\mu$ m.

### Attachment No. 7



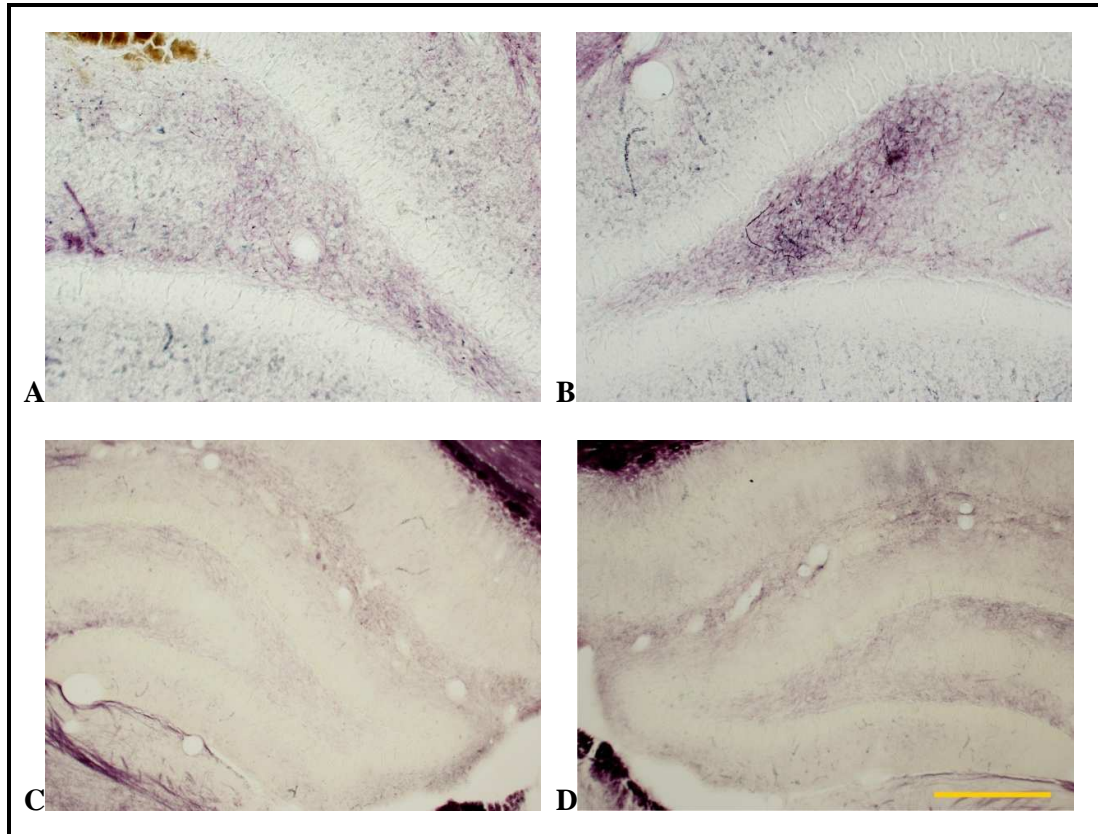
Black-Gold stained hippocampal coronal sections from a control animal. Most of the CA3 hippocampal area projections are present septally (white arrows) projections from the CA1 hippocampal area are emitted ventrally (black arrows). Scale bar= 500  $\mu$ m.

## Attachment No. 8



Black-Gold staining of the hippocampus of an ET-1 treated animal. There is an overt loss of black-Gold stained fibers in the stratum oriens and radiatum (arrows) of the CA1 hippocampal area (A), as compared to the contralateral hemisphere (B). Scale bar= 500  $\mu\text{m}$ .

## Attachment No. 9



Regional characterization of damage to the white matter in the hilus, as seen with Black-Gold labeling in the hippocampus of a P12 ET-1 treated animal (A, B) and a P25 ET-1 treated animal (C, D). There is a pronounced loss of myelinated fibers in the hilus of the ipsilateral hippocampus (A, C) as compared to the contralateral hilus (B, D). Scale bar= 100  $\mu$ m.